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Temporal Organization of Behavioral States through Local Neuromodulation in C. elegans

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TEMPORAL ORGANIZATION OF BEHAVIORAL STATES THROUGH LOCAL NEUROMODULATION IN *C. ELEGANS*

A Dissertation Presented

By

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Dedicated to my father Dipankar Banerjee and my mother Alokananda Banerjee

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"I learned this, at least, by my experiment: that if one advances confidently in the direction of his dreams, and endeavors to live the life which he has imagined, he will meet with a success unexpected in common hours."

— Henry David Thoreau, Walden: Or, Life in the Woods

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ABSTRACT

Neuropeptide signaling play critical roles in maintaining distinct behavioral states and orchestrating transitions between them. However, elucidating the mechanisms underlying neuropeptide modulation of neural circuits *in vivo* remains a major challenge. The nematode *Caenorhabditis elegans* serves as an excellent model organism to study neuropeptide signaling mechanisms encoded in relatively simple neural circuits. We have used the *C. elegans* egg-laying circuit as a model to understand how neuropeptide signaling modifies circuit activity to generate opposing behavioral outcomes. *C. elegans* egg-laying behavior is composed of alternating cycles of two states – short bursts of egg deposition (active phases) and prolonged periods of quiescence (inactive phases). We have identified two neuropeptides (NLP-7 and FLP-11) that are locally released from a group of neurosecretory cells (uv1) and coordinate the temporal organization of egg-laying by prolonging the duration of inactive phases. These neuropeptides regulate activity within the core circuit by inhibiting serotonergic transmission between its individual components (HSN motorneurons and Vm2 vulval muscles). This inhibition is achieved at least in part, by reducing synaptic vesicle abundance in the HSN synaptic regions. To identify potential downstream signaling components that mediate the actions of these neuropeptides, we have performed a forward genetic screen and have identified a strong candidate. In addition, we are trying to identify the receptor(s) of these neuropeptides by using a candidate gene approach. Together, we demonstrate that local neuropeptide signaling maintains the periodicity of distinct behavioral states by regulating serotonergic transmission in the core neural circuit.

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LIST OF ABBREVIATIONS

Nomenclature

ACh	acetylcholine
C.	*Caenorhabditis* genus
ChR2	channelrhodopsin-2
cAMP	cyclic adenosine monophosphate
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EEG	electroencephalogram
Ga	G-protein alpha subunit
GPCR	G-protein coupled receptor
GABA	gamma aminobutyric acid
GFP	green fluorescent protein
Glu	glutamate
HA	histamine
HisCl	histamine gated chloride channel
5-HT	5-hydroxytryptamine / serotonin
kb	kilobase
L1 - L4	*C. elegans* larval stages 1 – 4
N2	*C. elegans* wild type strain N2 (Bristol)
NE	norepinephrine
NGM	nematode growth media

OP50	*Escherichia coli* strain OP50
P	promoter
RFP	red fluorescent protein
ROI	region of interest
RGS	regulator of G-protein signaling
WT	wild type
YFP	yellow fluorescent protein

Symbols and Units

A	anterior
C	Celsius
g	gram
l	litre
m	meter
ml	milliliter
mg	milligram
mm	millimeter
µm	micrometer
M	molar
mM	millimolar
min	minutes
nM	nanomolar

ns	not significant
P	posterior
s, sec	second
SEM	standard error of mean
t	time
W	watt
W/m^2	watt per square meter
α	alpha
β	beta
γ	gamma

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Figure I-2B:

Roscoe O. Brady Jr., Neeraj Tandona, Grace A. Mastersb, Allison Margolis, Bruce M. Cohen, Matcheri Keshavan, Dost Ongür. (2016) Differential brain network activity across mood states in bipolar disorder. Journal of Affective Disorders. http://dx.doi.org/10.1016/j.jad.2016.09.041

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Schafer, W. F. (2006). Genetics of egg-laying in worms. *Annual Review of Genetics*, *40*, 487–509. http://doi.org/10.1146/annurev.genet.40.110405.090527

Figure III-1:

Frooninckx, L., Rompay, L., Temmerman, L., Sinay, E., Beets, I., Janssen, T., … Schoofs, L. (2012). Neuropeptide GPCRs in *C. elegans*. *Frontiers in Endocrinology*, *3*(DEC), 1–18. http://doi.org/10.3389/fendo.2012.00167.

CHAPTER I: INTRODUCTION

"We are part of nature, a product of a long evolutionary journey. To some degree, we carry the ancient oceans in our blood. … Our brains and nervous systems did not suddenly spring into existence without long antecedents in natural history. That which we most prize as integral to our humanity - our extraordinary capacity to think on complex conceptual levels - can be traced back to the nerve network of primitive invertebrates, the ganglia of a mollusk, the spinal cord of a fish, the brain of an amphibian, and the cerebral cortex of a primate."

- Murray Bookchin

Defending the Earth: A Dialogue Between Murray Bookchin and Dave Foreman

Composition of animal behavior

Animal behaviors are often temporally organized into distinct alternating states. Transitions between these component states or modifying their duration provides a facile mechanism to improve their chances of survival and reproductive success. At the appropriate time, animals must either continue with a particular phase of behavior or switch to a different phase. This can be triggered by a variety of environmental conditions or by internal homeostatic cues. For example, many foraging animals alternate between periods of active foraging spells and migration between feeding sites (Owen-Smith et al., 2010). The timing and duration of feeding and migration depends both on external factors such as food availability and quality, risk of predation and internal states such as metabolic costs or arousal levels. High quality of food during spring leads to animals spending shorter foraging times that alternates with longer periods of food search whereas low food quality during dry summer season ensures longer foraging spells interspersed by shorter search intervals (Heitschmidt and Stuth, 1991) (Figure I-1). Transitions between behavioral states can also be probabilistic and can occur without any trigger stimuli. For example, freely behaving mice in home cages typically alternate between active states characterized by feeding and patrolling and inactive states of rest and sleep (Goulding et al., 2008). A key strategy for survival relies on animals being able to rapidly modify the timing and duration of component behavioral states in response to changes in the environment as well as internal physiology.

Behavioral states have been shown to be associated with distinct patterns of activity in specific regions of the mammalian brain. For example, *in vivo* recordings from

neocortex in mice have revealed state-dependent changes in network activity associated with sleep-wake behavioral pattern. During sleep, there is a predominance of slow waves representing Up and Down states in the local network. Transition to wakefulness is associated with termination of Down states and enhancement of higher frequency rhythms (Zagha and McCormick, 2014) (Figure I-2A). Behavioral states are also shown to be correlated with global changes in activity in the human brain. fMRI studies have revealed changes in functional connectivity across different brain regions in bipolar disorder patients undergoing distinct mood states – mania and euthymia (Brady Jr. et al., 2016) (Figure I-2B).

All behaviors ranging from simple reflexes to sleep, mood, and higher cognitive tasks result from the activity of neural circuits within the nervous system. How do neural circuits coordinate transitions between behavioral states and maintain their duration for appropriate time intervals? In addition, how do neural circuits alter their activity patterns in response to changes in external conditions and internal cues to modify the duration of component behavioral states? Understanding the cellular mechanisms and identifying the molecular candidates involved in altering neural circuit activity and establishing distinct behavioral states remains one of the major challenges in neurobiology.

Neuromodulation of behavior

How are alternating behavioral states encoded within anatomically hardwired neural circuits? The first idea came from early studies in the mollusc *Tritonia,* which demonstrated that the same network of neurons could generate alternating behavioral paradigms – a defensive withdrawal reflex and an escape swimming response (Getting

and Dekin, 1985). These studies raised the intriguing possibility that neural circuits may display an inherent functional flexibility that allow them to adapt to changing conditions. The most detailed analysis of potential mechanisms underlying such flexibility within neural circuits came from studies on the crustracean stomatogastric ganglion (STG). It is a motor circuit comprising of ~30 neurons associated with rhythmic feeding behavior. Two sub-circuits within the STG motor circuit generate distinct rhythmic outputs – the faster pyloric rhythm (once every second) and the slower gastric rhythm (every 5-10 seconds). The functioning of the neurons involved in generating these two different rhythms are not strictly dependent on their anatomical connectivity within their respective sub-circuits. Neurons within the pyloric circuit can switch inputs to join the gastric circuit and vice-versa or change their firing patterns in a context-dependent manner (Weimann and Marder, 1994; Hooper and Moulins, 1989).

The molecular candidates that orchestrate reconfiguration of circuits or activity changes within circuit components has been largely attributed to a class of molecules called neuromodulators. In contrast to classical neurotransmitters that are released at synaptic sites and act on fast-acting ionotropic receptors, neuromodulators are largely secreted in a hormonal fashion and may act either locally or may travel long distances to act on distant target cells (Figure I-3). In a majority of cases, the receptors that mediate the actions of neuromodulators are G-protein coupled receptors (GPCRs). GPCRs have slower kinetics of action compared to ionotropic receptors and have much longer lasting effects. As a result, neuromodulatory signaling is more suited to generate and maintain long-lasting behavioral states. In several studies, neuromodulators have been reported to establish distinct behavioral states and mediate transitions between them. For

example, several neuromodulators such as acetylcholine (ACh), norepinephrine (NE), serotonin (5-HT), histamine (HA), and glutamate (Glu) have been implicated in the transition from sleep to wakefulness in mice (Figure I-2). Neuropeptides constitute the largest class of neuromodulators and have been reported to establish distinct behavioral states. For example, the agouti-gene related neuropeptide have been shown to promote feeding behavior (Aponte et al., 2011). In addition, vasopressin has been demonstrated to play critical roles in decision-making that underlies social behavior (Pitkow et al., 2001).

To decipher neuromodulatory effects on neural circuits and behavior, it is critical to understand the mechanisms of neuromodulation at the systems, cellular and molecular levels.

Mechanisms of neuromodulation

Studies in invertebrates such as lobster and *Aplysia* have shed light on how neuromodulatory signaling alter the functioning of neural circuits. The principles of neuromodulation are complex – single modulators may exert their effects on multiple neurons and activities of multiple channels or single neurons may be modulated by several neuromodulators. The two major mechanisms by which neuromodulators modify the functional connectivity of neural circuits are by (1) modulation of synapses and (2) modulation of neuronal excitability.

One simple mechanism of synapse modulation is modification of synaptic strength. Neuromodulators can either strengthen or weaken synaptic transmission between components within a circuit according to behavioral needs (Figure I-3A). For example,

serotonin can functionally silence synapses in the crustracean STG, while dopamine can unmask normally silent synapses (Johnson et al., 1994).

Neuromodulators can also change synaptic dynamics within a circuit. Modulation of short-term synaptic plasticity (STP) can act as a gain-control mechanism leading to changes in synaptic strength in response to repetitive presynaptic activity (Rothman et al., 2009; Abbott et al., 1997) (Figure I-3A). Studies on the *Aplysia* gill and siphon reflex in response to touch stimulus have shed light on neuromodulatory effects on synaptic dynamics (Hawkins et al., 2006). The functional sensorimotor connectivity responsible for the reflex action normally habituates but is sensitized by serotonin signaling triggered by pairing touch with a noxious tail shock. Serotonergic modulation leads to increase in the width of presynaptic action potential and enhanced neurotransmitter release (Kandel, 2001; Klein et al., 1982).

Responses to presynaptic inputs or intrinsic activity within neurons critically depend on the presence of ion channels that regulate neuronal excitability. Early studies have shown that neuromodulators may alter the gating properties of voltage-gated ion channels (Kaczmarek and Levitan, 1986) and also sometimes, change their unitary conductances (Pfeiffer-Linn and Lasater, 1996). In this way, neuromodulators can have profound effects on membrane excitability of neurons. Neuromodulatory effects on membrane excitability may be straightforward or more complex. For instance, neuromodulators may either alter excitability to lead to a linear increase or decrease of activity or may give rise to distinct patterns of activity by effecting excitability in a non-linear fashion (Figure I-3B). The classical mechanism of neuromodulation of ion channels can be attributed to binding of neuromodulators to G-protein coupled receptors which in

turn activate second messengers triggering the action of kinases and phosphatases that modify ion channel proteins. However, other pathways may also contribute such as direct gating by cyclic nucleotides (Podda and Grassi, 2013; He et al., 2014) or G proteins (Dascal, 2001), or direct and indirect effects of tyrosine kinases (Ahn et al., 2007; Lee et al., 2013). Different neuromodulators can affect distinct ion channels in the same neurons or different modulators can have converging effects on the same channel. Neuromodulators may also modify the properties of other types of channels. For instance, the two-pore domain K^+ channels involved in leak conductance in many neurons are subject to modulatory effects (Talley et al., 2000).

Modifications of circuit dynamics and circuit composition by synaptic modulation and altering neuronal excitability by neuromodulators allows for adaptability of circuit functioning in different behavioral contexts.

Challenges in studying neuromodulatory effects on behavior

Several well-demonstrated studies in higher organisms have indicated that neuromodulators can have profound effects on behavioral outcomes and play critical roles in establishing stable behavioral states. Neuromodulators have also been implicated in mediating the transition between opposing behavioral phases. However, the complexity of neural circuits in vertebrates and mammals have limited the quest for decoding the underlying mechanisms responsible for neuromodulation of circuits.

Remarkable progress has been made by investigating neuromodulatory mechanisms in the context of relatively simple neural circuits of crustaceans and

mollusks. Although such systems offer several technical advantages, most studies have been performed using dissected specimens and isolated neurons. In addition, such studies have relied on application of exogenous neuromodulators.

One of the major challenges in the field is to bridge the gap between neuromodulatory action on circuits *in vivo* and the generation of distinct behavioral states. It is critical to investigate the action of neuromodulators on neural circuits where they are endogenously released and dissect neuromodulatory mechanisms in intact freely behaving animals. Such studies would enable us to link *in vivo* activation of specific neuromodulatory signaling pathways with corresponding behavioral outcomes.

Caenorhabditis elegans

Over the past few decades, *C. elegans* has served as an excellent model organism for advancing the field of neurobiology. It is easy to maintain under laboratory conditions and has a short life-cycle of 3 days. It has a relatively simple nervous system consisting of 302 neurons and the anatomical connectivity of its nervous system has been fully elucidated by serial-electron microscopy (White et al., 1986). It is also amenable to powerful genetic analysis and this coupled to a short generation time, enables rapid production of transgenic animals and experimental analysis. *C. elegans* has a transparent cuticle that allows fluorescent imaging and optogenetics in intact living animals. Additionally, several molecular candidates and signaling pathways are conserved in higher organisms.

Even with a simple nervous system, *C. elegans* exhibits a diverse range of behaviors ranging from simple motor behaviors like feeding, locomotion, egg-laying to sensory behaviors like chemosensation and mechanosensation. The known anatomical connectivity of its nervous system coupled with ease of genetic manipulations enables dissection of behaviors at the cellular and molecular level.

Neuropeptide signaling in C. elegans

The *C. elegans* genome harbors approximately 119 neuropeptide precursor genes and encodes a vast array of about 265 neuropeptides. These are subdivided into three major families depending on the presence of conserved sequence motifs. The first family constitutes the FMRFamide-like peptide (*flp*) gene family that consists of 70 different peptides encoded by 33 precursor genes. The second family comprises about 40 insulin-like peptides (*ins*) encoded by 40 precursor genes. The third family consists of peptides without sequence similarity to either FMRFamide or insulin-like peptides, and are called neuropeptide-like proteins (*nlp*). This consists of the largest group of about 155 neuropeptides encoded by 46 precursor genes (Janssen et al., 2010).

Similar to mammals, mature neuropeptides in *C. elegans* are generated by posttranslational processing and modification of precursor molecules. Typically, the neuropeptide precursor is cleaved C-terminal to mono, di, or tribasic residues by proprotein convertase enzymes (EGL-3 / KPC-2). Basic amino acids are eliminated by carboxypeptidases E (EGL-21) to generate pro-neuropeptides. These are further modified by addition of specific motifs (such as amide groups) to yield mature peptides.

These active peptides are stored in dense-core vesicles which may be released either at synaptic sites or extrasynaptically in a humoral fashion. Several proteins have been reported to be involved in the transport and release of dense-core vesicles. These include the motor protein UNC-104, the calcium-dependant activator protein UNC-31, the protein kinase I PKC-1, and the protein tyrosine phosphate-like receptor IDA-1. (Hall and Hedgecock, 1991; Zahn et al., 2004; Sieburth et al., 2007).

Neuropeptide signaling have been demonstrated to modulate several different behaviors in *C. elegans*. One of the most well-studied examples is the food-related aggregation behavior. Some wild type strains forage on a bacterial lawn in a solitary fashion whereas others clump together into aggregates. Neuropeptide signaling mediated by FLP-21 peptides and its receptor NPR-1 have been reported to promote solitary feeding and inhibit social feeding (de Bono and Bargmann, 1998). Another example comes from neuropeptide modulation of food search behavior. When wild type worms are removed from food, they undergo an exploratory behavior in search of food. This behavior is characterized by an increase in the number of high angle body bends and orientations to maximize the chances of finding food in the local environment. This phase of exploratory behavior is mediated by neuropeptides encoded by the *nlp-12* gene and *nlp-12* mutants are defective in food search (Bhattacharya et al., 2014).

Thus, *C. elegans* can be an useful model to study the modulatory actions of neuropeptides *in vivo* and deciphering their specific effects on neural circuits where they are endogenously released.

C. elegans egg-laying behavior

C. elegans are self-fertile hermaphrodites producing their own sperm and oocytes that undergo internal fertilization to produce embryos. Embryos are stored in the uterus and typically a wild type animal retains around 15 eggs *in utero.* Specialized sex-specific muscles contract to open the vulva and allow successful deposition of eggs. Under favorable conditions, egg-laying behavior displays a specific temporal pattern. Typically, the animal stochastically alternates between two distinct behavioral states - short bursts of egg-laying (active phase) ranging from 1 – 5 minutes in duration and prolonged periods of quiescence (inactive phase) of about 20 minutes on average (Figure I-5B, C).

The exact reason(s) for maintaining such a temporal organization of egg-laying is largely unclear although several reasons can be speculated. First, after fertilization, animals typically retain their eggs *in utero* until they reach a certain threshold of multicellular development (usually >20 cell stage embryos). Arresting egg-laying activity for long periods may allow the eggs to reach the threshold and thus prevent exposure of premature embryos in the environment. Second, this temporal pattern of egg-laying may allow spatial dispersal of eggs and prevent local overcrowding and competition for food.

C. elegans has the ability to dramatically alter its rate of egg-laying in response to changes in the environment. Egg-laying activity reduces significantly in the absence of food (Trent, 1982). Similarly, mechanical stimulation, high osmolarity of the medium and high levels of carbon-dioxide have been shown to reduce egg-laying rates (Sawin, 1996; Horvitz, 1982; Fenk and de Bono, 2015).

Thus, the temporal organization of egg-laying behavior into two opposing behavioral states and its sensitivity to changes in external context provide a useful model to study neuromodulatory control of behavior.

Anatomy and functional connectivity of the C. elegans egg-laying circuit

C. elegans egg-laying is controlled by a relatively simple neuromusculature and the anatomical connectivity between its components has been elucidated (Figure I-6A, B). A total of sixteen muscles are likely involved in the control egg-laying but of them, the four vm2 vulval muscles are the most critical. They are arranged in a cross-shape with their apical ends attached to the vulva and are electrically coupled to each other by gap junctions. They are the only muscles receiving significant synaptic input from neurons and their ablation leads to complete termination of egg-laying suggesting that they play a critical role in vulval contraction during egg-laying. The vm2 muscles are electrically coupled to vm1 class of vulval muscles. These muscles do not receive significant synaptic input and also do not dramatically alter egg-laying when ablated. There are also eight uterine muscles surrounding the anterior and posterior arms of the uterus. These muscle are believed to constrict the uterus during egg-laying thereby pushing eggs out of the vulva. Ablation of these muscles do not result in any obvious changes in egg-laying. Two hermaphrodite-specific neurons (HSNs) are critical for regulating egg-laying behavior. The HSN cell bodies are located posterior to the vulva and extend a process ventrally into the ventral nerve cord and then anteriorly towards the nerve ring. At close proximity to the vulva, the HSNs make synapses with the vm2 vulval muscles. The HSNs promote egg-

laying at least in part, by releasing serotonin (Horvitz, 1982; Desai, 1988), although it may also use acetylcholine (Duerr, 2001) and one or more neuropeptides (Schinkmann and Li, 1992; Kim and Li, 2004). Six VC motorneurons in the ventral nerve cord can be subdivided into two groups – vulval-proximal VC4 and VC5, and vulval-distal VC1-3 and VC6. VC4 and VC5 exclusively make synapses with the vulval muscles and other VCs. The other VCs make fewer synapses with the vulval muscles than VC4 and VC5, but direct synaptic output to the body wall muscles and the D-class GABAergic motorneurons. The VCs are cholinergic (Duerr, 2001) and also express at least RFamide peptide (Schinkmann and Li, 1992). They also stain weakly for serotonin but do not express the serotonin biosynthesis enzyme tryptophan hydroxylase suggesting that they may locally reuptake serotonin from neuromuscular junctions between the HSNs and vulval muscles. The role of VCs in regulating egg-laying has not been unambiguously elucidated but several evidences suggest that they may be involved in vulval muscle contraction by cholinergic signaling mediated by acetylcholine receptors expressed in vulval muscles (Trent et al., 1983; Weinshenker et al., 1995; Waggoner et al., 2000).

Another group of cells known as uv1 cells do not make synapses with the rest of the egg-laying circuit, and are located at the junction between the uterus and the vulval epithelium, in close proximity to the egg-laying neuromusculature. These cells appear to function as neurosecretory cells and express the neuromodulator tyramine and the FMRFamide class of neuropeptides (Alkema, 2005; Kim and Li, 2004). The exact role of the uv1 cells in regulating egg-laying is largely unclear. The expression of candidate neuromodulators indicates that uv1 cells may play an important role in the temporal control of egg-laying.

The simplicity of the neuromusculature and anatomical connectivity of the egg-laying circuit would potentially serve as a good system to study the role of neuropeptides in modulating circuit activity and generating alternating behavioral outcomes.

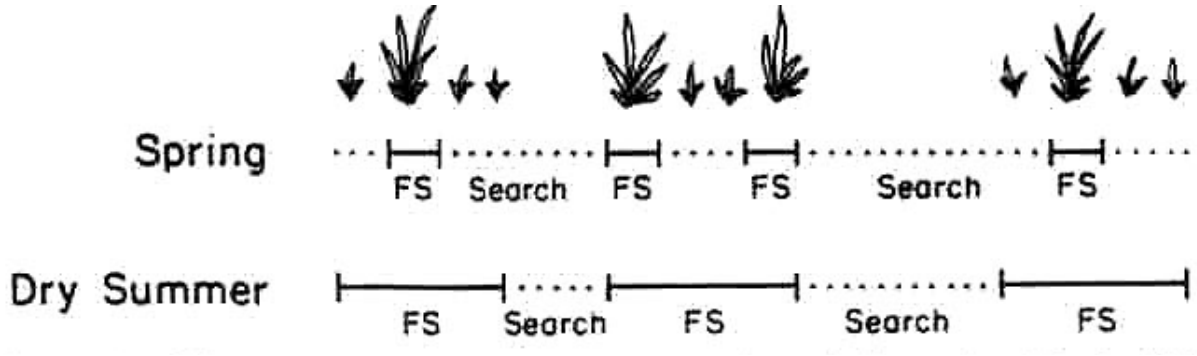

Figure I-1: Distinct behavioral states of foraging behavior

Effects of seasonal quality of plants on feeding behavior of herbivores. High quality of food in spring ensures shorter feeding times at foraging sites (FS) with longer periods of search between foraging sites. Conversely, low plant quality in dry summer season prolongs feeding times at foraging sites and reduces search intervals between sites.

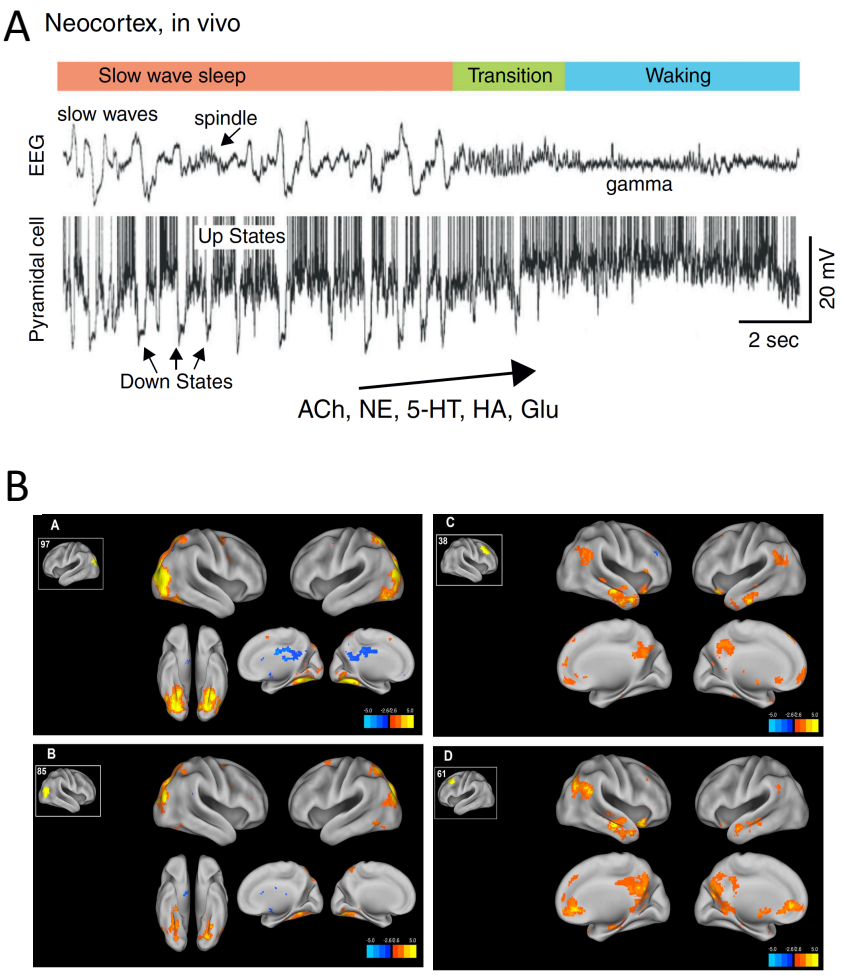

Figure I-2: Distinct patterns of brain activity correlate with different behavioral states. (A) State-dependent changes in activity within the cortex in mice. Slow wave sleep is associated with slow waves in the EEG and occurrence of Up and Down states in the local cortical field. Transition to wake state is accompanied by abolition of Down states and an increase in higher frequency rhythms (gamma waves). Several neuromodulators have been implicated in this transition. (B) Differences in whole brain functional connectivity in bipolar disorder patients undergoing bipolar mania vs bipolar euthymia. Each inset shows the regions of interest (ROIs) showing significant difference in functional connectivity in mania vs euthymia.

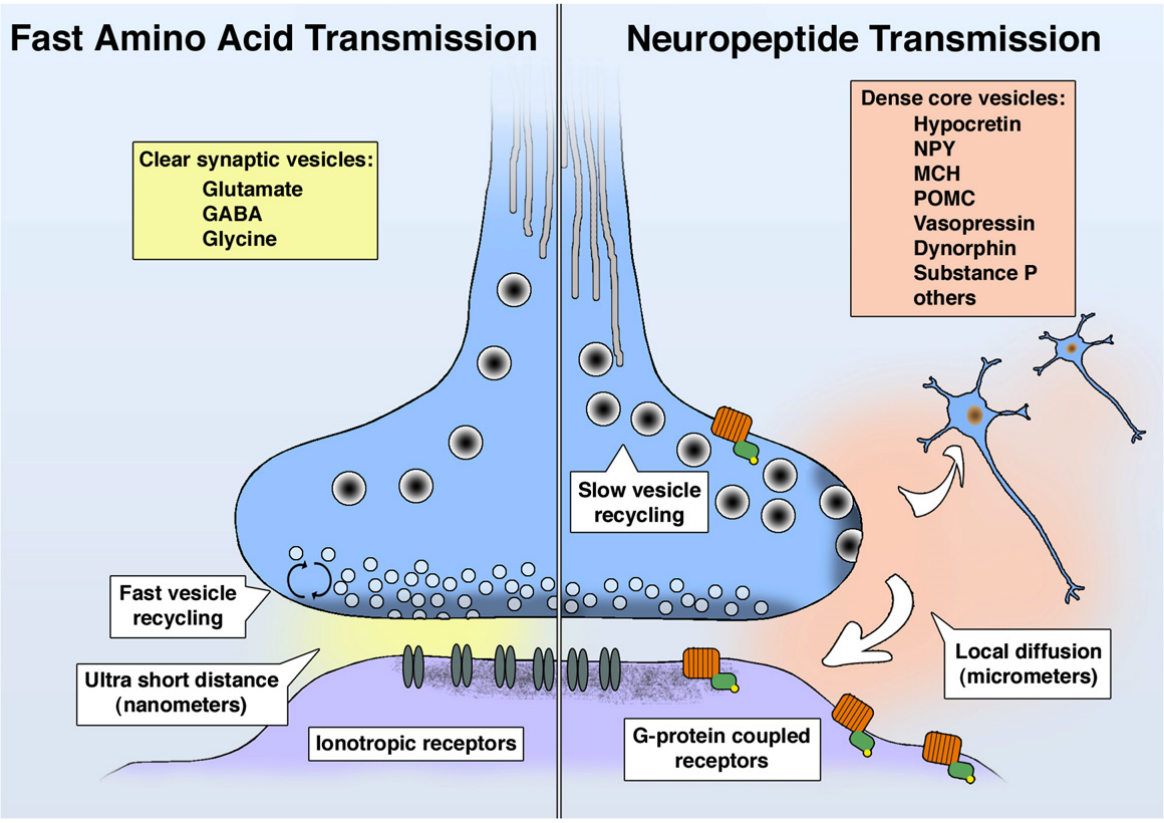

Figure I-3: Comparison of fast-acting neurotransmitters and slow-acting neuromodulators. Neurotransmitters are packaged in synaptic vesicles, released at synapses and act locally on ionotropic receptors. Neuromodulators such as neuropeptides, are typically packaged into dense-core vesicles which may act locally or may travel over long distances to act on G-protein coupled receptors to mediate their effects.

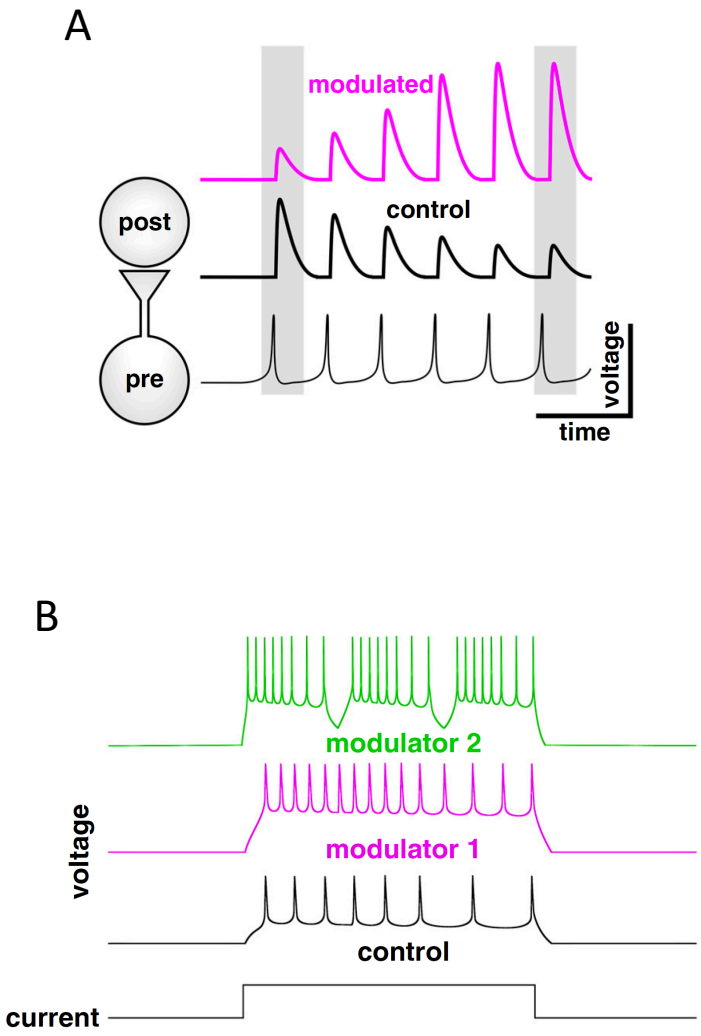

Figure I-4: Mechanisms of neuromodulation. (A) Neuromodulators can affect synaptic strength by modifying response to a single presynaptic input (left gray box) or short-term synaptic plasticity by modulating responses to sustained presynaptic inputs (right gray box). (B) Neuromodulators may modify membrane excitability either in a straightforward manner (modulator 1) or in a complex non-linear fashion (modulator 2).

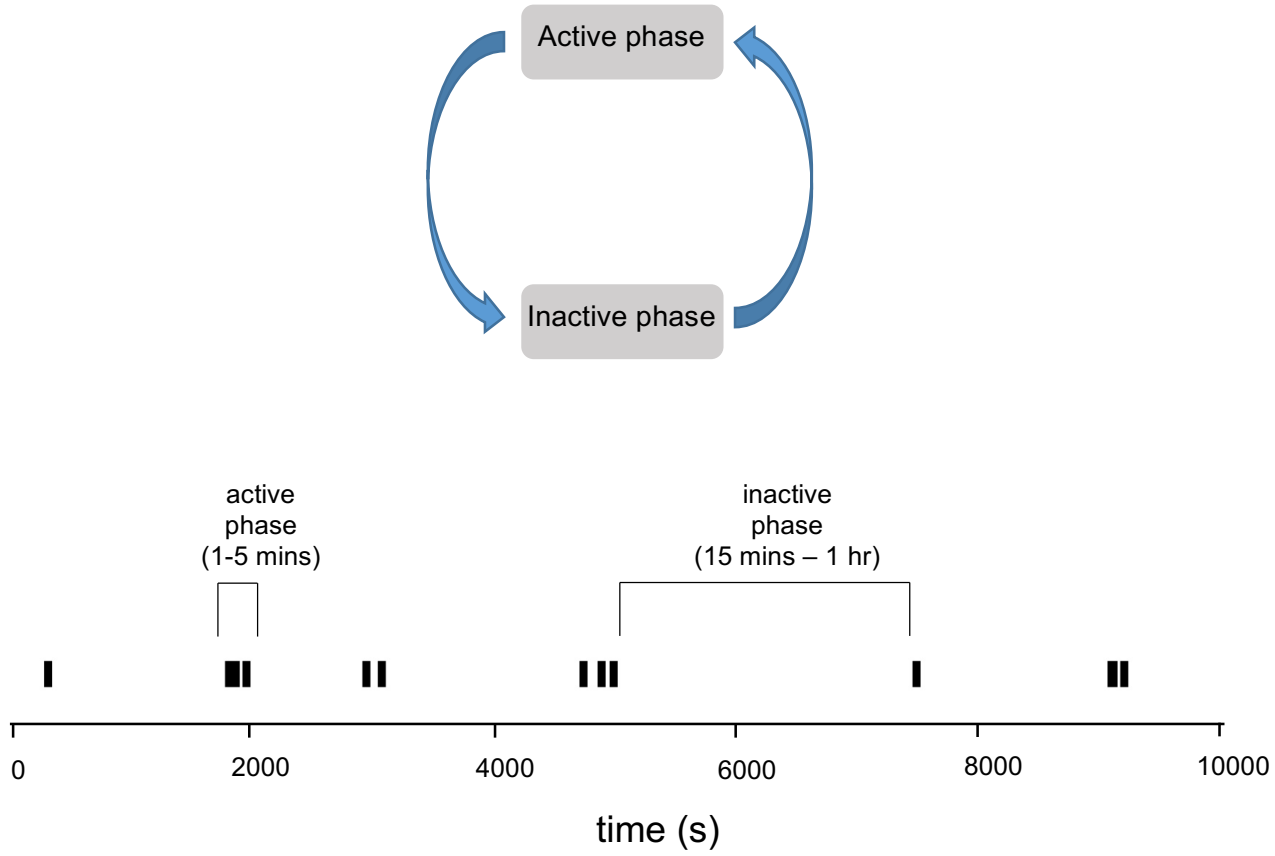

Figure I-5: *C. elegans* egg-laying is composed of distinct behavioral states.

(A) Cartoon showing the opposing phases of egg-laying behavior. Egg-laying activity is composed of alternating cycles of active and inactive phases. (B) Temporal organization of egg-laying behavior. Typically, under laboratory conditions, short bursts of egg-laying activity (active phases) are interspersed with prolonged periods of quiescence (inactive phases).

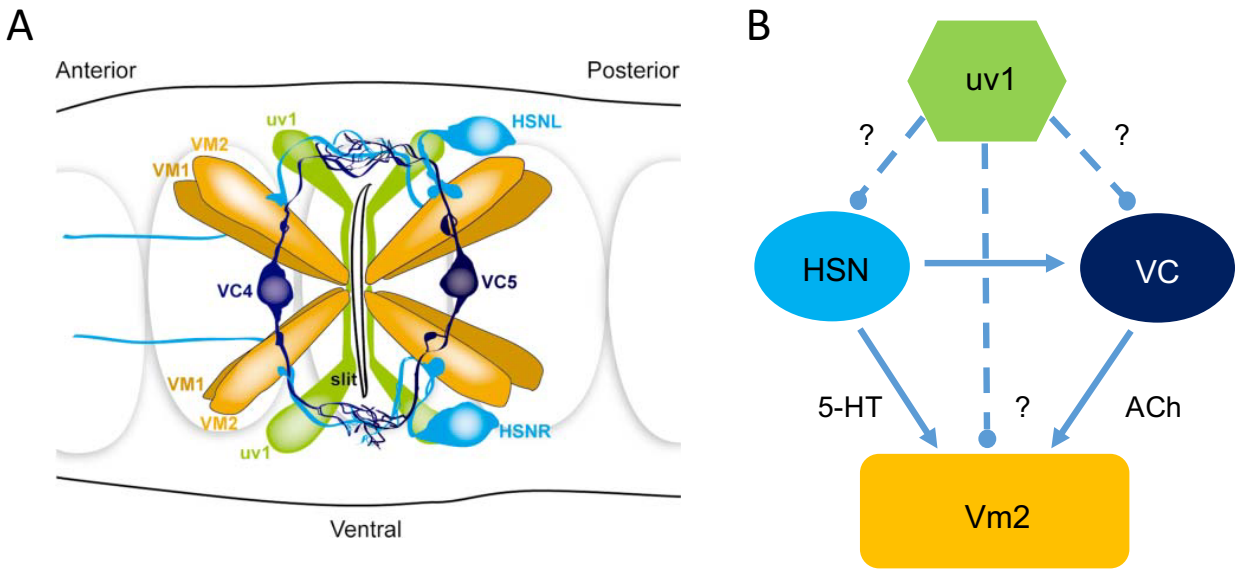

Figure I-6: Anatomy and connectivity of the *C. elegans* egg-laying circuit.

(A) Anatomy of the egg-laying neuromusculature (Schafer, 2006). See text for details. (B) Schematic showing connectivity within components of the egg-laying circuit. The HSNs make synaptic connections onto Vm2 vulval muscles and the VC5 motor neurons. Serotonin release from the HSNs is required for egg-laying. Cholinergic VC4 and VC5 motor neurons make synaptic connections onto the vulval muscles. The VCs might also inhibit the HSNs through putative extrasynaptic signaling. The synaptic connections of VCs onto muscle are primarily excitatory. Neurosecretory uv1 cells express several neuromodulators with potential for regulating egg-laying activity but do not have direct postsynaptic targets. Circles represent putative inhibitory connections while arrows denote excitatory connections. Solid lines depict known synaptic connections while dashed lines depict putative extrasynaptic signaling. Connections based on Collins and Koelle, 2013; Schafer, 2006; White et al., 1986; Zhang et al., 2008.

Thesis outline

This thesis demonstrates how neuromodulatory signaling mechanisms are encoded within a neural circuit *in vivo* to generate a specific temporal pattern of behavior. The model system I used here is the egg-laying behavior of the nematode *C. elegans.* Egg-laying behavior of *C. elegans* follows a temporal organization where the animal typically undergoes alternating cycles of two distinct behavioral states – short bursts of active egg-laying (active phase) followed by prolonged periods of quiescence (inactive phase). While the connectivity of the circuit and the molecular signals responsible for successful deposition of eggs is well characterized, the mechanisms governing the temporal pattern of egg-laying remain unclear.

In Chapter II, we demonstrate that altering the activity of a group of neurosecretory cells (uv1) located in close proximity to the egg-laying neuromusculature, results in disruption of the timing of egg-laying events. We also identify two neuropeptides (NLP-7 and FLP-11) that are expressed in the uv1 cells and are required for the modulatory effects of uv1 cells on egg-laying behavior. By monitoring egg-laying activity in freely behaving animals, we show that these neuropeptides synergistically inhibit egg-laying by prolonging the duration of inactive phases. By cell-specific overexpression, we demonstrate that local release of NLP-7 and FLP-11 peptides from uv1 cells is required for their inhibitory effects. Using a combination of optogenetics, pharmacology as well as genetic loss-of-function and gain-of-function tools, we show that NLP-7 inhibits serotonergic transmission between the HSNs and vulval muscles. Our imaging results

reveal that this inhibition is at least in part, achieved by reducing synaptic vesicle abundance in the HSN presynaptic compartment.

Neuropeptides typically mediate their effects by binding to G-protein coupled receptors (GPCRs). In Chapter III, we make an attempt to identify potential receptor(s) and downstream signaling components that are responsible for mediating the inhibitory effects for these neuropeptides. Overexpression of NLP-7 (NLP-7 OE) led to robust inhibition of egg-laying. We are using a candidate-gene approach using GPCR loss-of-function mutants to identify suppressors of the egg-laying defect of NLP-7 OE animals. Additionally, we have also performed a forward genetic screen to identify genes that may act downstream of NLP-7 signaling. We have identified a potential candidate that strongly suppresses the NLP-7 OE egg-laying defect. Future experiments would reveal the identity of this candidate and its role in neuropeptide signaling.

Neuromodulation allows flexibility to structurally hardwired circuits by modifying their activity patterns in response to external conditions as well as internal states of the animal. In this thesis, I have used the *C. elegans* egg-laying circuit as an *in vivo* system to demonstrate that local neuromodulation mediated by neurosecretory cells modifies neurotransmission within a core circuit to shape the temporal pattern of behavior. This thesis also provides insights into potential signaling mechanisms mediated by GPCRs that underlie the modulatory actions of neuropeptides. Knowledge of neuropeptide signaling mechanisms in simple circuits can be extended to understanding the basis of neuromodulatory effects on behavior in more complex systems.

PREFACE TO CHAPTER II

The work presented in this chapter demonstrates how neuromodulators alter the activity pattern of neural circuits to shape the temporal organization of behavior. Neuromodulatory actions are superimposed on anatomically hardwired circuits to help them to adapt and change their firing patterns in response to the changing environment or internal physiology. The effects of several neuromodulators allow the same set of neurons within a circuit to display alternate activity patterns and thus generate distinct behavioral states. In this chapter, we have dissected neuromodulatory signaling mechanisms *in vivo* that regulate the timing of egg-laying behavior in *C. elegans*. Under favorable laboratory conditions, egg-laying activity alternates between two distinct behavioral states – short bursts of egg deposition (active phases) followed by prolonged periods of quiescence (inactive phases). In this chapter, neuromodulatory signaling mediated by two neuropeptides are shown to govern the timing of egg-laying by prolonging the duration of inactive phases. These neuropeptides modify the activity of the neural circuit responsible for egg-laying by inhibiting serotonergic transmission between motorneurons and muscles. This chapter links the *in vivo* actions of specific neuromodulators on circuits where they are endogenously released with the generation of opposing behavioral outcomes.

Navonil Banerjee performed the behavioral, optogenetic and pharmacological experiments and analyzed all data. Michael Gorczyca performed the imaging experiments of HSN synapses and cell bodies. Navonil Banerjee and Michael Francis designed the experiments and co-wrote the manuscript. Navonil Banerjee, Raja Bhattacharya and

Kevin M Collins generated transgenic strains. All authors commented on the manuscript. This work was accepted for publication in *PLoS Genetics* at the time of this thesis presentation.

Local neuropeptide signaling modulates serotonergic transmission to shape the temporal organization of *C. elegans* egg-laying behavior

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Short title: Neuromodulatory control of opposing behavioral states

Abstract

Animal behaviors are often composed of distinct alternating behavioral states. Neuromodulatory signals are thought to be critical for establishing stable behavioral states and for orchestrating transitions between them. However, we have only a limited understanding of how neuromodulatory systems act *in vivo* to alter circuit performance and shape behavior. To address these questions, we have investigated neuromodulatory signaling in the context of *Caenorhabditis elegans* egg-laying. Egg-laying activity cycles between discrete states–short bursts of egg deposition (active phases) that alternate with prolonged quiescent periods (inactive phases). Here using genetic, pharmacological and optogenetic approaches for cell-specific activation and inhibition, we show that a group of neurosecretory cells (uv1) located in close spatial proximity to the egg-laying neuromusculature direct the temporal organization of egg-laying by prolonging the duration of inactive phases. We demonstrate that the modulatory effects of the uv1 cells are mediated by peptides encoded by the *nlp-7* and *flp-11* genes that act locally to inhibit circuit activity, primarily by inhibiting vesicular release of serotonin from HSN motor neurons. This peptidergic inhibition is achieved, at least in part, by reducing synaptic vesicle abundance in the HSN motor neurons. By linking the *in vivo* actions of specific neuropeptide signaling systems with the generation of stable behavioral outcomes, our study reveals how cycles of neuromodulation emanating from non-neuronal cells can fundamentally shape the organization of a behavioral program.

Author Summary

Animals have robust mechanisms in place to shape their behavior in a manner that is beneficial both for their survival and for the survival of their progeny. A class of signaling molecules known as neuropeptides have been implicated in driving transitions between behavioral states but we have only a limited understanding of how neuropeptide signaling modulates neural circuit activity in vivo to elicit alternate behavioral outcomes. Egg-laying behavior in the model system *C. elegans* cycles between clusters of egg-laying and prolonged inactive periods. This temporal organization provides for spatial dispersal of eggs, presumably benefiting progeny by limiting local overcrowding and competition for food. Here we uncover a novel neuromodulatory mechanism that shapes the timing of egg-laying behavior. Specifically, we find that neuromodulatory signaling from a group of non-neuronal cells specifies transitions between active and inactive phases of egg-laying by regulating neurotransmitter release from the motor output neurons of the egg-laying circuit. Advancing our knowledge of how neuropeptides and other modulators act in the context of the circuits in which they are endogenously released will be critical in ongoing efforts to understand how alternate behavioral states, for example those underlying mood or arousal, are encoded.

Introduction

Animals have robust mechanisms in place to shape their behavior in a manner that is beneficial both for their survival and for the survival of their progeny. In many cases behaviors are composed from discrete, opposing behavioral states. Examples of behaviors that are organized in this manner range from the motor programs underlying feeding or locomotion to more complex behaviors such as mood or arousal (Ben Arous et al., 2009; Fujiwara et al., 2002; Osborne et al., 1997; Saper et al., 2010; Young and Dulcis, 2015) . In each of these cases, animals adapt their behavior in response to either changes in environmental conditions or internal physiological signals by modifying the duration of component behavioral states.

How the nervous system establishes the duration of particular behavioral states and executes transitions between them, however, is largely unclear. Neuromodulators such as neuropeptides are attractive candidates for orchestrating these processes (de Lecea, 2012; Flavell et al., 2013; Laurent et al., 2015; Waggoner et al., 1998; Waggoner et al., 2000; Wang et al., 2013). Pharmacological, biochemical, and electrophysiological studies have shed light on how neuropeptide signaling can modify the activity of neural circuits (Bargmann, 2012; Bargmann and Marder, 2013; Kupfermann and Weiss, 2001; Marder, 2012; Palmer and Kristan, 2011; Taghert and Nitabach, 2012). Neuropeptide signals typically activate or refine patterns of neural activity that are generated from the actions of fast-acting transmitters by altering cellular excitability or by modifying the efficiency of synaptic communication between neurons. However, precisely linking *in vivo* activation of specific neuromodulatory systems with changes in circuit performance and the generation of alternate behavioral outcomes remains a challenge. Uncovering basic

principles by which neuromodulators influence circuit activity and behavior in more simple systems provides an important framework for tackling similar questions in the mammalian brain. In particular, the neural circuit controlling egg-laying behavior in the nematode *Caenorhabditis elegans* provides a relatively simple and experimentally tractable system to address these questions.

Under favorable conditions, *C. elegans* egg-laying behavior cycles probabilistically between two alternative states: active phases containing short bursts of egg-laying and prolonged periods of quiescence (inactive phases) (Collins et al., 2016 ; Garcia and Portman, 2016; Schafer, 2006; Waggoner et al., 1998). This temporal organization of egg-laying allows for spatial dispersal of eggs, and may be beneficial for survival of progeny by limiting local overcrowding and competition for food. The core circuitry and signaling machinery controlling egg-laying is well described, but the mechanisms responsible for this temporal organization have remained unclear. Egg-laying occurs when specialized sex-specific muscles contract, opening the vulva and allowing for deposition of eggs. Two serotonergic hermaphrodite-specific neurons (HSN) and two cholinergic ventral C class motor neurons (VC4 and VC5) make excitatory synaptic contacts onto the Vm2 muscles (White et al., 1986). Egg-laying active states are associated with serotonin release from the HSNs that increases vulval muscle excitability (Figures 1A, 3A) (Hapiak et al., 2009; Schafer, 2006; Shyn et al., 2003; Trent et al., 1983; Waggoner et al., 1998).

Four neurosecretory cells, known as uv1 cells, are located in close proximity to both HSN processes and the egg-laying musculature, forming a ring through which eggs pass as they exit the uterus and vulva (Newman et al., 1996). The uv1 cells do not form synaptic connections with the egg-laying circuitry, but express candidate

neuromodulators (e.g. FMRFamide peptides, tyramine), raising the possibility that secretion of neuromodulators from the uv1 cells may allow for temporal coordination of egg-laying activity (Alkema et al., 2005; Schinkmann and Li, 1992). By linking acute cell-specific activation to alternate behavioral outcomes, we demonstrate that uv1 secretion of neuropeptides encoded by the *nlp-7* and *flp-11* genes inhibits HSN-vulval muscle transmission, setting the duration of inactive phases during egg-laying. Unlike the other neurotransmitter systems described to date involved in the inhibition of egg laying, NLP-7 and FLP-11 peptides do not strictly depend on $G_{i/o}$ G-protein coupled receptor (GPCR) signaling. Instead, inhibition involves redundant functions of $G_{i/o}$ and the gustatory receptor-like protein EGL-47. Our studies support a model where neuropeptide signaling controls the temporal organization of opposing behavioral states by altering synaptic vesicle abundance in motor output neurons.

Results

Altering uv1 activity affects the timing of egg-laying events

To begin to address the role of the uv1 cells in neuromodulatory control of egg-laying, we first asked whether manipulations that alter uv1 activity affect the retention of eggs *in utero*, an indirect measure of egg-laying activity. Wild type adults typically retain about 15 eggs *in utero* under normal laboratory conditions. We used cell-specific expression of a histamine-gated chloride channel (HisCl1) to selectively hyperpolarize the uv1 cells (Pokala et al., 2014). The *ocr-2* promoter region and 3' UTR regions provide for selective expression in the uv1 cells and a few additional head and tail neurons (Jose et al., 2007). Using these regulatory regions to drive HisCl1 expression, we silenced uv1 cells with exposure to exogenous histamine (6 hrs) and then measured the number of eggs retained in the uterus (Figure 1B). Transgenic animals expressing HisCl1 without exposure to exogenous histamine retain a similar number of eggs to wild type. Histamine-mediated hyperpolarization of the uv1 cells causes a significant reduction in the number of eggs *in utero* (31%, $p < 0.0001$), consistent with an increased rate of egg-laying. To investigate this further, we next asked whether hyperpolarization of the uv1 cells altered the temporal organization of egg-laying events by monitoring egg-laying in freely moving animals (Figure 1C, D). Under favorable growth conditions, bouts of egg-laying (clusters) are separated by prolonged inactive phases (15-60 mins) in which eggs are retained in the uterus. We found that uv1 hyperpolarization causes a significant shift towards shorter inactive phases (Figure 1D).

We next sought to determine whether uv1 activation is sufficient to inhibit egg-laying. To address this question, we expressed channelrhodopsin (ChR2) in uv1 cells using the

regulatory regions of *ocr-2* as above. Light stimulation immediately following the initial egg-laying event of an active phase (see Methods) significantly delays subsequent egg-laying events, and also significantly reduces the total number of egg-laying events within an active phase (Figure 2)(Movie S1). For example, under control conditions a majority (~80%) of animals show a delay between the first and second egg-laying events within an active phase of <20 s (light stimulation, -ATR) (Figure 2B). This proportion is reduced dramatically (to around 10%) when uv1 cells are activated (light stimulation, +ATR). In addition, a significant fraction of uv1 activated animals (~33%, compared with 11% of controls) halt egg-laying for >5 mins following light stimulation, likely indicating termination of the active phase (Figure 2B). Of the animals that progress to a second egg-laying event, the average time interval between the first and second egg-laying events increases from roughly 12 s under control conditions to nearly 38 s with uv1 depolarization. Approximately 30% of the animals tested lay 5 or more eggs within an active phase under control conditions, but active phases with >5 events are not observed with uv1 depolarization (Figure 2C). Moreover, about 60% of uv1-activated animals lay ≤2 eggs after light stimulation compared to just 22% of animals under control conditions. Taken together, our findings provide evidence that uv1-mediated inhibition of egg-laying promotes periods of quiescence in the egg-laying program and plays a key role in setting their duration.

NLP-7 and FLP-11 neuropeptides are expressed in the uv1 cells and synergistically inhibit egg-laying behavior

The dense core vesicle marker IDA-1 is expressed in the uv1 cells, and we noted that an IDA-1::GFP translational fusion produces punctate fluorescence in the uv1 cells, consistent with prior reports documenting the presence of secretory vesicles in uv1 cells (Schinkmann and Li, 1992; Zahn et al., 2001; Zahn et al., 2004) (Figure S1A). These findings suggest that neuromodulatory signals are likely to be important for uv1 regulation of egg-laying. We therefore investigated the expression of several neuropeptide precursors–potential dense core vesicle cargoes–in the uv1 cells. We used a reporter construct in which coding sequence for the fluorescent protein mCherry preceded by the SL2 splice leader was added to the native genomic sequence encoding selected neuropeptide precursors. This bicistronic vector drives expression of the precursor and the mCherry reporter under the control of native regulatory sequences. Amongst the candidates tested, the NLP-7 precursor was of particular interest, producing mCherry fluorescence in cells neighboring the vulva (Figure S1B). We also observed mCherry fluorescence in head and tail neurons, consistent with a prior report (Nathoo et al., 2001). The mCherry fluorescence near the vulva clearly labels the uv1 cells and the VC4 and VC5 motor neurons, and overlaps with that of IDA-1::GFP (Figure 3B and S1C). The *nlp-7* gene encodes four mature peptides that belong to the SFamide family (Figure S1D), but functions for these peptides have only recently begun to be elucidated (Mills et al., 2012; Park et al., 2009; Park et al., 2010). We also confirmed expression of a second neuropeptide precursor, *flp-11*, in the uv1 cells using a *Pflp-11::*GFP transcriptional reporter. Consistent with previous reports (Kim and Li, 2004; Turek et al., 2016), we observed *flp-11::*GFP fluorescence in head and tail neurons, and also prominently in the uv1 cells and VC4 and VC5 motor neurons (Figures S2A and 3B). While *flp-11::*GFP

expression is evident in at least one uv1 cell of all adults tested, and remains detectable throughout adulthood, the number of uv1s labeled is somewhat variable across individuals. The *flp-11* neuropeptide precursor is predicted to give rise to four mature peptides of the FMRFamide class (Figure S2B) and *flp-11* expression in RIS neurons located in the head has been previously implicated in the control of sleep (Turek et al., 2016). Our demonstration of *nlp-7* and *flp-11* expression in the egg-laying circuit raises the interesting possibility that local release of these peptides may regulate circuit performance and egg-laying behavior.

To explore the above possibility, we first investigated egg-laying behavior in *nlp-7* and *flp-11* single deletion mutants. *nlp-7(lf)* and *flp-11(lf)* animals were not significantly different from the wild type with respect to the number of eggs retained in the uterus (Figure 3D). Similarly, single deletion of *nlp-7* or *flp-11* does not significantly advance the developmental stage at which embryos are laid (1-8 cell stage: wild type 10%, *nlp-7(lf)* 10%, *flp-11(lf)* 6%, n>80 eggs for each genotype), suggesting that loss of either single neuropeptide precursor does not appreciably affect the rate of egg-laying.

Neuropeptides often work in parallel pathways to mediate specific behavioral outcomes. To test whether NLP-7 and FLP-11 may be acting cooperatively to regulate egg-laying, we investigated egg-laying behavior in *nlp-7(lf);flp-11(lf)* double mutants. Strikingly, we found that the double mutants retain significantly fewer eggs *in utero* than either wild type or the respective single mutants (Figure 3C, D). In addition, double mutants lay a significantly higher percentage of eggs in the early cell stage (1-8 cell stage) than wild type (wild type: 10%; *nlp-7(lf);flp-11(lf)*: 58%, n>100 eggs for each genotype)(Figures 5D and S3). Transgenic expression of wild type *nlp-7* or *flp-11* in *nlp-*

7(lf);flp-11(lf) double mutants under control of their respective native promoters reverses these effects (Figure 3D). Thus, our findings suggest that either class of peptides, if present at sufficient levels, is capable of slowing the rate of egg-laying. Indeed, transgenic expression of *nlp-7* increases the retention of eggs *in utero* beyond wild type levels, suggesting that a chronic increase in circulating levels of NLP-7 peptides may be sufficient to produce prolonged inhibition of the egg-laying circuit and deficits in egg-laying.

To address whether specific peptide expression in in the uv1 cells is sufficient to rescue constitutive egg-laying in *nlp-7(lf);flp-11(lf)* mutants, we expressed either *nlp-7* or *flp-11* under the control of *ocr-2* regulatory sequence. uv1-specific expression of either peptide precursor completely reverses hyperactive egg-laying, supporting the idea that the uv1 cells are an important endogenous source of these peptides for regulation of egg-laying (Figure S3). We therefore next tested whether uv1-mediated inhibition of egg-laying is dependent on *nlp-7* and *flp-11* neuropeptides by measuring egg-laying responses to uv1 photostimulation in *nlp-7(lf);flp-11(lf)* double mutants (Figure 3E, F)(Movie S2). As described in Figure 2, uv1 stimulation in control animals increases the interval between egg-laying events and reduces the number of events during an active phase. For depolarization of uv1 cells in *nlp-7(lf);flp-11(lf)* double mutants, the interval between consecutive egg-laying events is significantly decreased compared to uv1 stimulation in controls. Moreover, 90% of *nlp-7(lf);flp-11(lf)* double mutants perform ≥3 egg-laying events following light stimulation compared with 40% of controls that perform ≥3 events (Figure 3E, F). These results support the idea that secretion of NLP-*7* and FLP-11 peptides in response to stimulation of the uv1 cells is sufficient to inhibit egg-laying.

However, we note that the effects of uv1 stimulation are not completely reversed in *nlp-7;flp-11* double mutants, consistent with the possibility that other modulatory signals may also contribute to uv1 regulation of egg-laying.

To determine which aspects of the egg-laying behavioral program may be subject to modulation by NLP-7 and FLP-11 peptides, we monitored the timing of egg-laying events in freely moving animals. In particular, we quantified the time intervals between consecutive active phases of egg-laying (intercluster intervals) as well as intervals between individual egg-laying events within an active phase (intracluster intervals). The duration of intercluster intervals in *nlp-7(lf);flp-11(lf)* double mutants is strikingly reduced compared with wild type or either single mutant (Figure 4A, B). In contrast, the duration of intracluster intervals is largely unaffected. Together, these results support a model where coordinated actions of NLP-7 and FLP-11 slow the rate of egg-laying by prolonging the length of the inactive phase (i.e. delaying transitions to the active phase).

Local release of NLP-7 peptides from uv1 cells produces sustained inhibition of egg-laying

Our findings in the above rescue experiments prompted us to investigate whether increasing NLP-7 peptide levels may be sufficient to modify egg-laying behavior and, over time, lead to an egg-laying defective phenotype. To explore this possibility further, we expressed the *nlp-7* genomic sequence (including ~3.5 kb promoter region and 3' UTR) at high levels in otherwise wild type animals. We found that egg-laying behavior is severely disrupted in animals stably expressing the *nlp-7* transgene (NLP-7 OE; *ufIs118*) (Figure 5A, B). In particular, these animals retain significantly more eggs *in utero* than the

wild type (Figure 5B), and lay a significantly higher percentage of eggs in later stages of development (~95% in comma to 3-fold stage compared with 0 in the wild type)(Figure 5D, E). In contrast, overexpression of *flp-11* does not cause significant retention of eggs, but does rescue constitutive egg-laying in *nlp-7;flp-11* double mutants as noted above (Figure 3). The retention of eggs *in utero* with *nlp-7* overexpression provides evidence that chronic increases in NLP-7 peptide levels are sufficient to suppress egg-laying.

In order to investigate the time course over which NLP-7 may act in the control of egg-laying we next asked whether acute *nlp-7* expression is also sufficient to alter egg-laying. To address this question, we expressed *nlp-7* under the control of a heat-inducible promoter. At a constant temperature of 20°C, animals carrying this transgene do not show any obvious egg-laying defects (Figure 5C(i)) compared to non-transgenic controls. In contrast, exposing transgenic animals to a brief heat shock stimulus (33°C for 30 mins) produces a striking decrease in the rate of egg-laying. This decrease is evident within 2 hours following heat shock and lasted for a period of approximately four hours (Figure 5C(ii)). Further, these decreases are reversible, with animals increasing their egg-laying rates approximately 4 hrs following heat shock, such that total egg-laying approaches control levels by 6 hrs after heat shock (Figure 5C(ii)). These findings demonstrate that an acute rise in NLP-7 peptide levels is sufficient to block egg-laying in adult animals, and the time course of these effects suggests that the actions of NLP-7 are transient, consistent with the predicted modulatory effects of a neuropeptide.

We next pursued cell type-specific overexpression of *nlp-7* to decipher where peptide expression may be required for modulation of egg-laying. Our prior expression studies revealed that *nlp-7* is expressed in cells proximal to the vulva (uv1 and VC4/5) as

well as in neurons near the head and tail that are distal to the core egg-laying circuit. An analysis of the *nlp-7* promoter region revealed a 2.5 kb fragment that drove predominant expression in the head and tail neurons, but provided more limited expression in the egg-laying circuit (fluorescence in egg-laying cells was visible in roughly 60% of animals). Using this 2.5 kb promoter to drive expression, we investigated egg-laying in the group of animals where we were able to confirm *nlp-7* expression was restricted to head and tail neurons. Selective expression in head and tail neurons does not produce an appreciable difference in the developmental stage of embryos at the time of egg-laying (Figure 5F), indicating that expression in these neurons alone is not sufficient for modulation of egg-laying activity. We also analyzed egg-laying in the group of animals for which we observed *nlp-7* expression in both head and tail neurons and the egg-laying cells (uv1 as well as more variable expression in the VC4 and VC5 neurons) using the shorter 2.5 kb *nlp-7* promoter fragment. We noted a significant increase in the fraction of eggs laid at later developmental stages in these animals (Figure 5G). To decipher which cells may be most critical, we used cell-specific promoters to drive overexpression of *nlp-7*. Surprisingly, despite the spatial proximity of the VC4 and VC5 neurons to the HSNs and vulval muscles, specific expression of *nlp-7* in the VC neurons (*lin-11* promoter fragment) does not alter egg-laying (Figure 5H). In contrast, specific expression in the uv1 cells (using *ocr-2* promoter and 3' UTR) produces a striking shift in the developmental stage of eggs at the time of egg-laying (Figure 5I), similar to that observed using the 3.5 kb native *nlp-7* promoter. Together, our results support the idea that local NLP-7 release from uv1 cells is required for modulation of egg-laying activity. Further, the inability to elicit changes in

egg-laying activity with expression in neighboring VC neurons may point toward a specialized role for the uv1 cells within the circuit.

NLP-7 and FLP-11 reduce the activity of the egg-laying circuit

Synaptic release from the HSN neurons onto vulval muscles is a key factor in the control of egg-laying activity. Therefore, to begin to address the question of how the behavioral effects of peptide modulation may be encoded within the egg-laying circuit, we next investigated whether neuropeptide signaling altered responsiveness in this core egg-laying pathway. Prior work showed that photostimulation of transgenic animals that express ChR2 in the HSNs is sufficient to elicit an active period of egg-laying (Emtage et al., 2012) (Figure 6A). We therefore asked whether neuropeptide signaling affected bouts of egg-laying elicited by HSN photostimulation. We quantified egg-laying events in response to a 60 s period of light stimulation, measuring the time interval between the onset of light exposure and the initial egg-laying event as described previously (Emtage et al., 2012) (Movie S3). As expected, control animals expressing ChR2 respond in a dose-dependent manner to blue light stimulation–the time interval to the initial egg-laying event decreased with increasing light intensities (Figure 6B). Using a light intensity that produced an intermediate response in controls (20 W/m^2), we found that *nlp-7(lf);flp-11(lf)* double mutants are hypersensitive to light stimulation–there is a significant increase in the fraction of animals that respond to light stimulation over time compared with control animals or either single mutant (Figure 6C). In contrast, *nlp-7* overexpression completely blocks egg-laying responses to HSN photostimulation. Whereas almost 60% of wild type animals respond within 20 secs of blue light exposure, NLP-7 OE animals show no

response within the 60 s period of light exposure (Figure 6D). Likewise, higher light intensities (up to 200 W/m^2) do not elicit egg-laying responses in NLP-7 OE animals (none of the animals tested respond within 60 s, n=30). Thus, altering peptide levels can have profound effects in this core HSN-muscle pathway, suggesting that NLP-7 and FLP-11 peptides likely inhibit egg-laying behavior either by acting to inhibit neurotransmitter release from the HSN neurons or by reducing responsiveness of egg-laying muscles.

Elevating serotonin release or supplying exogenous serotonin reverses egg-laying defects caused by increased NLP-7

Based on our above optogenetic analysis, we used multiple approaches to explore mechanisms by which NLP-7 and FLP-11 peptides regulate HSN-muscle signaling and egg-laying. The HSNs are primarily serotonergic and we therefore first tested whether pharmacological approaches that alter serotonin levels would normalize egg-laying in NLP-7 OE animals. Prior work demonstrated that the egg-laying defects produced by serotonin deficiency are reversed by supplying exogenous serotonin (Desai and Horvitz, 1989; Trent et al., 1983). We found that serotonin exposure stimulates egg-laying similarly in both wild type animals and neuropeptide-deficient single mutants. Serotonin exposure elicits fewer egg-laying events in *nlp-7;flp-11* double mutants, likely due to the fact that these animals display constitutive egg-laying and retain fewer eggs *in utero* (Figure 3D). Strikingly, serotonin exposure also promotes egg-laying in NLP-7 OE animals, suggesting that egg-laying defects in these animals might arise through a deficiency in serotonin levels (Figure 7A). Consistent with this interpretation, we found that the serotonin reuptake inhibitor fluoxetine elicits far less egg-laying activity in NLP-7 OE animals

compared with wild type (Figure 7B). Fluoxetine exposure also elicits a reduced number of egg-laying events in *nlp-7;flp-11* double mutants compared with wild type, again likely because these animals retain fewer eggs *in utero*.

We next pursued several genetic approaches to ask whether HSN activity and serotonin release are regulated by neuropeptide signaling. We used available loss-of-function mutations in *egl-47* and *goa-1*, two genes that act primarily in the HSNs to inhibit egg-laying, to elevate HSN signaling (Mendel et al., 1995; Moresco and Koelle, 2004; Segalat et al., 1995; Shyn et al., 2003) (Figure 7C). *egl-47* encodes a seven transmembrane protein bearing homology to *Drosophila* gustatory receptors (Liu et al., 2010) while *goa-1* encodes Ga_o, the only inhibitory G-protein subunit expressed in the HSNs. Mutation of *egl-47* partially reverses NLP-7 inhibition of egg-laying (Figure 7D). Though loss of *goa-1* produces constitutive egg-laying in otherwise wild type animals, we did not observe appreciable differences in egg-laying between control NLP-7 OE animals and NLP-7 OE animals carrying a *goa-1(lf)* mutation. Combined mutation of *egl-47* and *goa-1* however completely reverses the effects of *nlp-7* overexpression, eliciting a constitutive egg-laying phenotype comparable to that of *goa-1(lf)* single mutants (Figure 7D). We next examined whether combined mutation of *egl-47* and *goa-1* in NLP-7OE animals restored responsiveness to HSN photostimulation. Egg-laying responses elicited by ChR2 activation of the HSNs approach wild type levels in these animals (Figure 7E), providing additional evidence that NLP-7 inhibition of egg-laying is reversible with increases in HSN signaling. Finally, we asked whether increasing muscle synaptic activation could suppress NLP-7 mediated inhibition of egg laying. Cholinergic transmission at synapses between VC neurons and egg-laying muscles operates in

parallel with HSN signaling onto muscles to control their excitability. Vulval muscles express acetylcholine receptors that are responsive to the nematode-specific cholinergic agonist levamisole, and exposure to exogenous levamisole stimulates egg-laying (Kim et al., 2001). We increased cholinergic synaptic activation of muscles by expressing a gain-of-function muscle acetylcholine receptor (L-AChR*(gf)*) (Bhattacharya et al., 2014). Transgenic expression of L-AChR*(gf)* in otherwise wild type animals increases egg-laying activity, producing a constitutive phenotype (Figure 7F). In contrast, expression of L-AChR*(gf)* in NLP-7 OE animals does not appreciably change the rate of egg-laying, indicating that elevated cholinergic synaptic activation of muscles cannot reverse the inhibition produced by increased levels of NLP-7 signaling. Interestingly, NLP-7 OE animals expressing the L-AChR*(gf)* transgene show enhanced egg-laying with a supply of exogenous serotonin (–5-HT: 0.4±0.3 eggs/hour; +5-HT: 12.8±1.9 eggs/hour, $p<0.0001$, t-test), consistent with the notion that the production or release of serotonin may be impaired in NLP-7 OE animals.

As noted above, FLP-11 expression is sufficient for rescue of constitutive egg-laying in *nlp-7;flp-11* double mutants, but overexpression does not elicit sustained inhibitory effects. Therefore, we were unable to pursue a similar genetic analysis of FLP-11 signaling. This could be due to a functional property of the FLP-11 signaling pathway or may indicate that our strategies for increasing neuropeptide expression were less effective for FLP-11. Nonetheless, our findings for NLP-7 argue that these peptides act primarily to control levels of serotonin secretion from the HSNs. Prior work showed that HSNs exhibit spontaneous Ca^{2+} oscillations even in the absence of synaptic inputs (Zhang et al., 2008). Therefore, our findings suggest a model where local neuropeptide

release from the uv1 cells may control circuit activity, in part by shaping the timing of vesicular serotonin release from the HSNs.

NLP-7 regulates synaptic vesicle abundance in the HSN neurons

To gain additional support for peptide regulation of HSN transmission, we next examined the distribution of synaptic vesicles at HSN synapses onto vulval muscles. Numerous previous studies have employed the synaptic vesicle marker synaptobrevin (SNB-1) to investigate alterations in the distribution of synaptic vesicles in the HSNs (Nonet, 1999; Shen and Bargmann, 2003; Shen et al., 2004; Tanis et al., 2008; Tanis et al., 2009). Therefore for our analysis, we used a transgenic strain in which GFP-tagged synaptobrevin (SNB-1::GFP) is stably coexpressed specifically in the HSN neurons with the fluorescent reporter DsRed2 (Tanis et al., 2008).

In wild type animals, the HSN process forms two to four synaptic varicosities within 10μm of the vulval opening where synaptic contacts with the Vm2 vulval muscles are located (Tanis et al., 2009). We did not observe significant changes in the morphological features of the HSN neurons in either of the neuropeptide single mutants or in *nlp-7;flp-11* double mutants. Likewise, the total synaptic volume (Figure S5A) and the number of synaptic varicosities (Figure S5B) within the HSN process are unaffected by single or combined deletion of the *flp-11* and *nlp-7* neuropeptide precursors, suggesting that the sizes of the synaptic region are not altered. However, we noted a significant increase in the intensity of SNB-1::GFP fluorescence in both *nlp-7(lf)* single mutants and *nlp-7(lf);flp-11(lf)* double mutants (Figure 8B), suggesting that synaptic vesicle abundance in the HSN neurons is increased in these animals. In principle, the increase in vesicle abundance

could affect either egg-laying activity during the active phase or the length of the quiescent period. Despite increased SNB-1::GFP, we did not observe a significant change in the number of egg-laying events/active phase for either *nlp-7(lf)* single mutants and *nlp-7(lf);flp-11(lf)* double mutants (wild type: 3.8±0.3; *nlp-7(lf)*: 4.1±0.3; *nlp-7(lf);flp-11(lf)*: 3.2±0.2), supporting the idea these peptides act primarily to increase the length of the quiescent period. Deletion of *flp-11* did not produce an appreciable difference in SNB-1::GFP intensity, suggesting that HSN regulation may be primarily mediated through NLP-7 peptides, while FLP-11 peptides may modulate egg-laying activity via another mechanism. Conversely, we noted a significant decrease in SNB-1::GFP fluorescent intensity in NLP-7 OE animals (Figure 8A, B), though the total volume of the synaptic region remained unchanged (Figure S5A). Finally, DsRed2 fluorescent intensity (expressed under the same promoter as SNB-1::GFP) in either the synaptic region or the HSN cell body is not significantly affected by combined peptide deletion or by NLP-7 overexpression (Figures 8C and S5E), providing evidence that the changes in SNB-1::GFP fluorescence we observe do not arise via transcriptional regulation of the reporter. Similarly, neither localization or expression of the active zone marker GFP::SYD-2 were appreciably altered by peptide deletion or overexpression (Chia et al., 2013) (Figure S6), providing additional evidence that synapse organization is not affected by altering neuropeptide signaling. In contrast, SNB-1::GFP fluorescent intensity in the HSN cell bodies is significantly reduced by NLP-7 overexpression, similar to the synaptic region (Figure S5C, D). Together, our analysis supports a model where neuropeptide secretion from the uv1 cells modulates egg-laying activity, at least in part by limiting the abundance of serotonergic synaptic vesicles at HSN synapses. As reduced synaptic vesicle

trafficking to synapses might be expected to lead to an accumulation of synaptic vesicles in the cell body, we propose that this regulation occurs via an alternate mechanism, perhaps through direct or indirect regulation of synaptic vesicle biogenesis.

Discussion

Neuromodulatory signaling enables hardwired circuits to maintain flexibility for generating alternate activity patterns and behaviors in response to either changes in environmental conditions or internal state of the organism. Gaining a mechanistic understanding of these processes *in vivo* however has remained a challenge. Our studies here demonstrate that neuropeptide modulation of neurotransmitter release controls the temporal pattern of *C. elegans* egg-laying activity. While prior work has provided examples of neuromodulatory control of egg-laying in response to external environmental factors (Fenk and de Bono, 2015; Ringstad and Horvitz, 2008; Waggoner et al., 2000; Zhang et al., 2008), our work demonstrates that neuromodulatory signaling is central for establishing the timing of egg-laying activity even under relatively constant favorable external conditions.

Prior work suggested that the neurosecretory uv1 cells may terminate egg-laying, perhaps by sensing the passage of eggs through the vulva (Collins et al. 2016; Jose et al., 2007). Our work supports this model and extends it in several important aspects. First, we show that activating or inhibiting the uv1 cells produces predictable changes in the periodicity of egg-laying. Second, we identify two neuropeptide precursors, NLP-7 and FLP-11, that are expressed in uv1 cells and work cooperatively to set the duration of inactive phases during egg-laying. For both neuropeptide precursors, specific expression in the uv1 cells is sufficient to slow the rate of egg-laying, suggesting that local secretion solely from uv1 cells can account for their effects *in vivo*. Third, we show that *nlp-7* overexpression produces robust and sustained inhibition of egg-laying, and provide evidence that this occurs through negative regulation of serotonin signaling from the HSN

neurons. Finally, genetic ablation or overexpression of *nlp-7* produce opposing effects on synaptic vesicle abundance in the HSN neurons. Genetic alteration of *flp-11* levels does not reproduce these effects, suggesting *flp-11* may act via another mechanism or target another cell type in the circuit. Together, our findings support a model where peptides derived from NLP-7 and FLP-11 are coordinately secreted from uv1 cells to modify egg-laying circuit activity and shape the temporal organization of egg-laying behavior. We propose that the secretion of NLP-7 and FLP-11 peptides from uv1 cells promote egg-laying quiescent periods by reducing serotonin release from the HSN neurons. This is achieved, at least in part, by regulating the abundance of serotonergic synaptic vesicles in the HSN neurons (Figure S7). Prior work showed that neuropeptides encoded by the *flp-1* gene promote the onset of egg-laying active phases (Waggoner et al., 2000). It is therefore appealing to speculate that the NLP-7/FLP-11 and FLP-1 neurotransmitter systems act antagonistically to shape egg-laying behavior. One intriguing possibility is that FLP-1 peptides primarily act in response to sensory stimuli (such as food availability) to alter an intrinsic rhythm established through the actions of NLP-7/FLP-11 signaling.

Our studies indicate that local neuropeptide signaling plays a central role in establishing behavioral rhythmicity during egg-laying. In particular, we find that cell-specific expression of *flp-11* or *nlp-7* is sufficient to rescue egg-laying defects or, in the case of *nlp-7*, produce sustained inhibition of egg-laying. Interestingly, cell-specific expression of *nlp-7* in VC motor neurons did not reproduce these effects, even though these neurons are located in close proximity to both HSN neurons and egg-laying muscles. This finding points toward the idea that the uv1 cells perform specialized neurosecretory functions in halting egg-laying. For example, uv1 cells may be specialized

for properly coordinating the timing of neuropeptide release with egg-laying activity or perhaps possess expanded capacity for neuropeptide release compared with neighboring neurons. Unfortunately, it has proven difficult to assess uv1 function directly. These cells are an essential structural component of the egg-laying anatomy, limiting the value of ablation studies or mutants deficient in uv1 development for addressing questions about function (Jose et al., 2007; Huang and Hanna-Rose, 2006). Prior studies exploited a dominant mutation in the TRPV channel *ocr-2* to strongly implicate uv1 cells in the regulation of egg-laying, but mechanistic interpretation of these studies is complicated by our limited understanding of the effects of the mutation on channel functional properties (Jose et al., 2007). We used expression of a histamine gated chloride channel to inhibit uv1 activity. Prolonged inhibition of the uv1 cells in adult animals significantly reduces the length of the inactive phase of egg-laying. A recent study found that calcium transients occur in uv1 cells immediately following egg-laying events during an active phase (Collins et al., 2016), suggesting that repetitive stimulation of uv1 cells may be required for inhibition of egg-laying. We speculate that each egg-laying event may trigger release from the uv1 cells, ultimately leading to local increases in peptide levels sufficient to terminate the active phase and delay re-entry into the subsequent active phase. This model is supported by several observations: (1) ChR2 depolarization of uv1 cells delays subsequent egg-laying events in an active phase and even terminates the active phase in a subset of animals. (2) The effects of uv1 stimulation are almost completely eliminated in double mutants lacking NLP-7 and FLP-11 peptides. (3) uv1-specific rescue or overexpression of *flp-11* and *nlp-7* demonstrate that high levels of either peptide are sufficient to inhibit egg-laying. Moreover, induced expression of NLP-7 peptides in adults

slows egg-laying within two hours and these effects begin to reverse by four hours. uv1 cells may share interesting parallels with oxytocin (OT) neurosecretory cells of the hypothalamus that are involved in the control of lactation. Secretion of OT (and subsequently milk ejection) is not temporally locked to the initiation of suckling but instead must surpass a certain threshold during suckling for a burst of OT release and milk ejection to occur (Armstrong, 2007; Stern and Armstrong, 1996). Similarly, we speculate that uv1 cells require a buildup of egg-laying events during an active phase to achieve sufficient levels of NLP-7 and FLP-11 peptides to terminate an active phase. These neuropeptides may also work in concert with other modes of uv1 signaling. For example, genetic disruption of tyraminergic signaling produces defects in egg-laying and prior work showed that tyramine was present in the uv1 cells (Alkema et al., 2005). As is the case for *nlp-7;flp-11* double mutants, the effects of tyramine deficiency are incomplete, suggesting that these signals may cooperate to alter circuit performance in complex ways.

While our work supports the idea that neuropeptide secretion from uv1 cells is key for proper temporal organization of egg-laying, the nature of the sensor that triggers peptide secretion remains an open question. All of our studies are performed under constant favorable external conditions. Therefore, peptide secretion from uv1 cells is likely triggered by sensing a change in the internal state of the organism, perhaps by sensing egg-laying status. Based on their anatomical position and expression of mechanosensory TRPV channels, the uv1 cells have been proposed to sense the mechanical load of the uterus (Jose et al., 2007). Consistent with this idea, recent evidence indicates that uv1 cells are mechanically deformed with the passage of eggs

through the vulva (Collins et al., 2016). Thus, mechanical deformation of the uv1 cells may directly trigger rises in intracellular Ca^{2+} and peptide secretion.

Our genetic analysis of *nlp-7* overexpression provides evidence that NLP-7 peptides act primarily at the level of the HSNs to inhibit egg-laying. We found that combined mutation of *egl-47* and *goa-1* completely suppress the effects of *nlp-7* overexpression. Prior work demonstrated that each of these genes mediate their effects on egg-laying by acting primarily in the HSN neurons (Moresco and Koelle, 2004), although *goa-1* expression in vulval muscles or uv1 cells may also contribute (Jose et al., 2007; Mendel et al., 1995). Nonetheless, it is difficult to conclusively demonstrate that the HSN neurons are the sole site of action for these peptides in the egg-laying circuit because high-affinity receptors for NLP-7 peptides have not yet been identified, and the cellular expression of several putative FLP-11 receptors remains uncharacterized. *egl-6* encodes the sole GPCR that has been previously demonstrated to show expression in the HSN neurons, but NLP-7 peptides do not activate EGL-6 *in vitro* (Ringstad and Horvitz, 2008). Though a precise mechanism for EGL-47 action remains unclear, our finding that mutation of *egl-47* partially restores egg-laying in NLP-7 OE animals raises the intriguing possibility that EGL-47 may act directly as a receptor for NLP-7 peptides. *egl-47* encodes a seven-transmembrane protein related to *Drosophila* gustatory receptors (Bargmann, 2006; Liu et al., 2010). While gain-of-function *egl-47* mutation inhibits egg-laying, our studies here are the first to demonstrate a phenotype associated with *egl-47* loss of function. Initial studies suggested that *egl-47* may be an orphan GPCR (Moresco and Koelle, 2004), while more recent studies suggest that EGL-47 inhibition of egg-laying may occur through activation of an associated chloride channel (Tanis et al., 2009). In addition, our findings

reveal that the egg-laying defects of NLP-7 overexpressing animals are completely suppressed only by combined mutation of *egl-47* and *goa-1*. This observation points towards the interesting possibility that multiple receptors might act downstream of NLP-7 signaling – perhaps EGL-47 in combination with GOA-1 ($G_{i/o}$)-coupled GPCR(s). Further characterization of EGL-47 and other potential NLP-7 receptors is an important subject for future studies. Several potential FLP-11 receptors have been identified to date (Frooninckx et al., 2012; Mertens et al., 2004; Mertens et al., 2006; Turek et al., 2016), but expression of these receptors in the egg-laying circuit has not yet been demonstrated. In our studies, FLP-11 expression in the uv1 cells was sufficient for rescue of constitutive egg-laying in *nlp-7;flp-11* double mutants, but overexpression did not produce obvious defects, preventing pursuit of a genetic suppressor approach to identify the GPCR(s) involved.

Our studies also address the question of how peptides secreted from the uv1 cells modulate egg-laying. Our analysis of SNB-1::GFP fluorescence in the HSN neurons offers support for the idea that NLP-7 peptides act, at least partially, by modulating HSN transmission onto egg-laying muscles. We observed an increase in SNB-1::GFP fluorescence at HSN synapses in *nlp-7(lf);flp-11(lf)* mutants, and a strong decrease with *nlp-7* overexpression. Because the total synaptic volume and number of varicosities are unaffected, we propose that these fluorescence changes reflect altered synaptic vesicle density. We therefore interpret our findings to indicate that peptidergic signaling alters synaptic vesicle abundance in the HSN neurons. This interpretation is supported by our HSN photostimulation studies where we found that *nlp-7(lf);flp-11(lf)* double mutants exhibit enhanced egg-laying responsiveness to HSN depolarization, while NLP-7 OE

animals are resistant. Prior work has identified mechanisms for intrinsic regulation of HSN activity (Branicky et al., 2014; Emtage et al., 2012; Tanis et al., 2009; Zhang et al., 2008). In particular, the potassium channel IRK-1 is important for inhibition of HSN activity by the EGL-6 GPCR. Although acting independently of EGL-6, NLP-7 and FLP-11 peptides might similarly exert their effects by inhibiting HSN activity. Our analysis of SNB-1::GFP fluorescence is not however consistent with the peptides acting solely via this mechanism. For example, if NLP-7 peptides inhibited egg-laying simply by decreasing HSN activity, we might expect an increase in SNB-1::GFP fluorescence at synapses due to a reduced rate of vesicular release. Surprisingly, we observe that NLP-7 mediated inhibition of egg-laying is associated with a strong decrease in SNB-1::GFP intensity, suggesting other processes contribute. One possibility is that chronic peptidergic inhibition of the HSNs leads to a compensatory decrease in the production of synaptic vesicles. Alternatively, neuropeptide signaling may directly modulate the rate of synaptic vesicle trafficking to HSN synapses. This possibility appears unlikely because we do not observe increased SNB-1::GFP intensity in the HSN cell bodies of NLP-7 OE animals, as would be expected if vesicles were accumulating in the HSN soma. Finally, peptidergic signaling may directly alter rates of HSN synaptic vesicle recycling or biogenesis. A previous report demonstrated that serotonin expression in the HSNs is altered by G-protein signaling (Tanis et al., 2008). Our findings may suggest the presence of a parallel mechanism in the HSNs for regulating vesicle production. This would be consistent with our observation that SNB-1::GFP intensity is decreased at both HSN synapses and in HSN cell bodies of NLP-7 OE animals. Interestingly, we also observed that SNB-1::GFP fluorescent intensity is significantly increased at HSN synapses in *nlp-7* single mutants, but these animals do

not display significant egg-laying defects and likewise are not hypersensitive to HSN photostimulation. These results argue that changes in HSN synaptic vesicle abundance alone cannot fully account for the actions of FLP-11 and NLP-7 peptides in the egg-laying circuit.

In summary, our results demonstrate a mechanism by which local neuropeptide secretion from a group of non-neuronal cells produces relatively long-lived inhibition of circuit activity and behavioral quiescence by modulating serotonergic transmission. HSN motor neurons exhibit spontaneous activity and serotonin release from the HSNs is associated with a transition from the inactive to active phase of egg-laying (Waggoner et al., 1998; Zhang et al., 2008). We propose that cycles of neuropeptide release from the uv1 cells reversibly inhibit HSN transmission and structure the timing of egg-laying by both promoting inactive phases and preventing rapid re-entry into active phases. More broadly, our studies elucidate a mechanism by which neuromodulatory systems modify circuit activity to specify the timing of transitions between opposing behavioral states. Advancing our knowledge of how neuropeptides and other modulators act in the context of the circuits in which they are endogenously released will be critical in ongoing efforts to understand how alternate behavioral states, for example those underlying mood or arousal, are encoded.

Materials and Methods

Strains

All nematode strains were maintained at 20°C on agar nematode growth media plates seeded with *E. Coli* OP50. The wild type reference animals for all cases are the N2 Bristol strain. The following strains were used or generated in this work: IZ2539: *ufIs160*[*Pocr-2::HisCl::SL2::GFP:ocr-2 3' UTR*, *Plgc-11::mCherry*], LX2047: *lite-1(ce314);lin-15(n765ts);vsIs189*[*Pocr-2::ChR::YFP::ocr-2* 3'UTR], IZ2634: *nlp-7(tm2984);flp-11(tm2706);lite-1(ce314);lin-15(n765ts);vsIs189,* NY2040: *ynIs40*[p*flp-11*::GFP], IZ1130: *lin-15(n765ts);ufEx378* [p*nlp-7::nlp-7::SL2::mCherry*], IZ1135: *nlp-7(tm2984)*, IZ2022: *flp-11(tm2706)*, IZ1589: *nlp-7(tm2984);flp-11(tm2706)*; IZ2263: *nlp-7(tm2984);flp-11(tm2706);ufEx792*[*Pnlp-7::nlp-7::nlp-7* 3'UTR, *Plgc-11::*GFP], IZ2407: *nlp-7(tm2984);flp-11(tm2706);ufEx876*[*Pflp-11::flp-11::flp-11* 3' UTR, *Plgc-11::*GFP], IZ2834: *nlp-7(tm2984);flp-11(tm2706);ufEx1099 [Pocr-2::nlp-7::ocr-2 3' UTR, Pocr-2::mCherry::ocr-2 3'UTR, Plgc-11::GFP],* IZ1823: *nlp-7(tm2984);flp-11(tm2706);ufEx582*[*Pocr-2::flp-11::ocr-2 3'UTR, Pocr-2::mCherry::ocr-2 3'UTR, Plgc-11::GFP*], LX1836: *lite-1(ce314);lin-15(n765ts);wzIs30*[p*egl-6::*ChR::YFP], IZ1587: *nlp-7(tm2984); lite-1(ce314);lin-15(n765ts);wzIs30*, IZ2008: *flp-11(tm2706); lite-1(ce314);lin-15(n765ts);wzIs30*, IZ2007: *nlp-7(tm2984);flp-11(tm2706); lite-1(ce314);lin-15(n765ts);wzIs30*, IZ1614: *ufIs118;lite-1(ce314);lin-15(n765ts);wzIs30,* IZ1236: *ufIs118 [Pnlp-7::nlp-7::nlp-7 3'UTR, Plgc-11::GFP]* , IZ2109: *ufEx730*[*Phsp16.2::nlp-7*], IZ1594: *ufEx521*[*Pocr-2::nlp-7::ocr-2 3'UTR, Plgc-11::mCherry*], IZ2006: *ufEx681*[*Plin-11::pes-10::nlp-7, Plin-11::pes-10::mCherry, Plgc-11::GFP*], IZ1692: *ufEx548*[*Pnlp-7(2.5kb)::nlp-7, Pnlp-7(2.5kb)::mCherry, Plgc-11::GFP*], RB850: *egl-47(ok677)V* , IZ1235: *egl-47(ok677);ufIs118*, IZ1324: *goa-1(n1134);ufIs118*, IZ2096: *egl-47(ok677), goa-*

1(n1134);ufIs118, MT2426: *goa-1(n1134),* IZ2683: *egl-47(ok677), goa-1(n1134),* IZ2474: *egl-47(ok677), goa-1(n1134);ufIs118;lite-1(ce314);lin-15(n765ts);wzIs30,* IZ236: *ufIs6 [Pmyo-3:: unc-38(V/S), Pmyo-3::unc-29(L/S), Pmyo-3:: lev-1(L/S)]*, IZ1274: *ufIs6;ufIs118,* LX967: *lin-15(n765ts);vsIs103,* IZ1881: *nlp-7(tm2984); lin-15(n765ts);vsIs103,* IZ1880: *flp-11(tm2706); lin-15(n765ts);vsIs103,* IZ1884: *nlp-7(tm2984);flp-11(tm2706); lin-15(n765ts);vsIs103,* IZ1812: *ufIs118; lin-15(n765ts);vsIs103,* BL5752: *inIs181(Pida-1*::IDA-1::GFP*);inIs182(Pida-1*::IDA-1::GFP*),* IZ2681: *inIs181;inIs182;ufEx378*, TV38: *wyIs12 [Punc-86:: GFP::SYD-2, Podr-1::GFP]*, IZ2818: *wyIs12;ufIs118*, IZ2819: *wyIs12;nlp-7(tm2984);flp-11(tm2706)*.

Molecular Biology and Transgenes

HisCl: A fragment containing the coding sequence of HisCl SL2 trans-spliced with GFP was ligated into pKMC281 (a gift from the Koelle lab) between the *ocr-2* promoter and the *ocr-2* 3' UTR to generate pNB41 (*pocr-2::HisCl::SL2::GFP::ocr-2 3'UTR*). This was injected (100 ng/μl) into N2 animals along with the coinjection marker pBB107 (*Plgc-11::mCherry*, 30 ng/μl). The resulting extrachromosomal array was stably inserted by X-ray integration to generate the transgene *ufIs160* and outcrossed four times to wild type.

nlp-7 expression pattern: pCL17 (*Pnlp-7::nlp-7::SL2::mCherry*, 50 ng/μl) was injected into *lin-15(n765ts)* animals along with the coinjection marker pL15EK (*lin-15(+)*, 50 ng/μl).

nlp-7 and flp-11 rescue: For *nlp-7* rescue, a 5104 bp PCR product containing the *nlp-7* promoter, genomic locus and the 3'UTR (-3448 bp to +1656 bp relative to the transcriptional start) was injected at 50 ng/μl into strain IZ1589 along with the coinjection

marker pHP6 (*Plgc-11::GFP,* 30 ng/μl). For *flp-11* rescue, a 3240 bp PCR product containing about 2.5 kb of the *flp-11* promoter, genomic locus and the 3'UTR (-2518 bp to +722 bp relative to the transcriptional start) was injected (100 ng/μl) into strain lZ1589 along with pHP6 (30 ng/μl).

NLP-7 overexpression: The NLP-7 OE strain (*ufIs118*) was generated by microinjection and subsequent X-ray integration of a 5104 bp PCR product (injected at 100 ng/μl) containing the *nlp-7* promoter (about 3.5 kb), genomic locus and the 3'UTR (-3448 bp to +1656 bp relative to the transcriptional start) along with pHP6 (*Plgc-11::GFP,* 50 ng/μl) as coinjection marker. The integrated strain was outcrossed five times with wild type.

Heat shock overexpression of nlp-7: pNB35 (*Phsp16.2::nlp-7*, 100 ng/μl) was injected into N2 animals along with pHP6 (*Plgc-11::GFP,* 30 ng/μl) as coinjection marker.

Cell specific overexpression of nlp-7

uv1: The *nlp-7* genomic sequence was ligated into pKMC281 (a gift from the Koelle lab) between the *ocr-2* promoter and the *ocr-2* 3' UTR to generate pNB28 (*Pocr-2::nlp-7::ocr-2 3'UTR*). A PCR product amplified from this plasmid was injected at 100 ng/μl into N2 animals together with pBB107 (30 ng/μl).

VC: A fragment containing the *lin-11* enhancer region fused to the *pes-10* basal promoter was PCR amplified from pDM4 and ligated into pENTR/D-TOPO vector to generate a gateway entry vector. It was recombined with gateway destination vectors containing the *nlp-7* genomic sequence and mCherry coding sequence to generate pNB14 and pNB15. pNB14 (*Plin-11::pes-10::nlp-7,* 100 ng/μl) was coinjected with pNB15 (*Plin-11::pes-10::mCherry,* 80 ng/μl) into N2 animals together with pHP6 (30 ng/μl).

Head and tail neurons: A 2537 bp fragment containing the *nlp-7* promoter (-2537bp relative to transcriptional start) was amplified from genomic DNA and ligated into pENTR/D-Topo vector to generate a Gateway entry vector. This was recombined to Gateway destination vectors containing the *nlp-7* genomic sequence and mCherry coding sequence to generate pNB24 (*Pnlp-7(2.5kb)::nlp-7*) and pNB25 (*Pnlp-7(2.5kb)::mCherry*) respectively. pNB24 (100 ng/μl) and pNB25 (50 ng/μl) were coinjected into N2 animals together with pHP6 (30 ng/μl).

Behavioral assays

Quantification of eggs in uterus. Age-matched adults were obtained by collecting late fourth larval stage (L4) animals and culturing at 20°C for 30 hrs (except in Figure 7 where synchronized adults 24 hrs after the L4 stage were used). For each strain analyzed, animals were individually dissolved in 25% sodium hypochlorite, and their eggs, which survived because of their protective eggshells, were quantified.

Embryo staging assay. To score the developmental stage of newly laid eggs, age-matched adults (30 hrs after the late L4 stage) were transferred to fresh nematode growth medium plates (15 animals per plate), allowed to lay eggs for 30 mins and removed. Eggs laid on the plates were examined by a high power dissecting microscope and categorized as described in Ringstad and Horvitz, 2008.

Analysis of temporal pattern of egg-laying. Single one-day old (24-30 hrs after the L4 stage) adult animals were placed individually on NGM agar plates and videotaped at room temperature for 4-5 hours at a rate 0.5 frames/s. Intervals between clusters of egg-laying (intercluster intervals) and intervals between individual egg-laying events within a cluster

(intracluster intervals) were determined by video analysis and manually scoring the timing of egg-laying events.

HisCl experiments. Histamine plates were made by diluting histamine dihydrochloride (Sigma Aldrich) into NGM agar media cooled to 55°C, to a final concentration of 50 mM. Plates were stored at 4°C and used within 2 weeks. To quantify the number of eggs *in utero,* age matched adults (24 hrs after the L4 stage) were placed on seeded NGM plates with or without histamine. After 6 hrs at room temperature, animals were individually bleached and the number of eggs retained *in utero* were counted. To analyze the timing of egg-laying events, one-day old adults were placed on histamine plates. After 1 hr of histamine exposure, animals were videotaped for 3-5 hrs while on the same histamine plates. The timing of individual egg-laying events was determined by manual video analysis. Control animals were treated similarly except that they were placed on plates lacking histamine.

Pharmacological assays. Individual staged adult animals were placed in 50 µl of low salt M9 buffer, or M9 containing 7.5 mg/ml serotonin or 0.5 mg/ml fluoxetine. After 1 hour in each condition, the number of eggs laid by each animal was quantified.

Channelrhodopsin experiments. Synchronized young adult animals were transferred to retinal plates for roughly 18 hours prior to experiment. To prepare retinal plates, an overnight culture of OP50 was mixed with all-*trans* retinal to a final concentration of 100 µM and 150 µl of the mix was seeded onto individual NGM agar plates. Plates were stored at 4°C under dark conditions and used within one week. Photostimulation experiments were conducted using a fluorescent dissecting microscope (Zeiss steREO Discovery.V12) equipped with a GFP filter set. Light intensities were measured at the

surface of assay plates using a light meter. For all photostimulation experiments, control and experimental animals were treated similarly except that controls did not receive retinal exposure.

uv1 stimulation. Single animals were monitored in the presence of retinal. After the first egg-laying event of an active phase, the animals were immediately exposed to blue light (100 W/m^2) for 5 seconds and subsequent egg-laying events were monitored. Videotaping was continued for 5-6 mins after the last egg-laying event to ensure the end of an active phase. We noted that photostimulation elicited an escape-like response in a subset of animals expressing the *Pocr-2::ChR2::ocr-2 3'UTR* transgene (Movie S1). The *ocr-2* promoter and 3' UTR used for ChR2 expression in the uv1 cells also drives expression in a small number of sensory neurons near the head (Jose et al., 2007). The avoidance behavior we observed likely arises due to activation of these neurons. Notably, this avoidance response also occurred *nlp-7(lf);flp-11(lf)* double mutants where we did not observe appreciable inhibition of egg-laying (Movie S2), indicating that the egg-laying and locomotory behaviors elicited by light stimulation are separable. NLP-7 and FLP-*11* peptides appear required for inhibition of egg-laying, but are not necessary for the avoidance response to light stimulation.

HSN stimulation. On the day of the experiment, single animals were transferred from retinal plates to OP50 plates (without retinal) and assayed immediately for egg-laying response to blue light exposure.

Heat shock overexpression experiments. Groups of 10 age-matched adult animals (picked at L4 stage on the day prior to experiments) were transferred to NGM agar plates seeded with OP50, wrapped in parafilm and submerged into a water-bath at 33°C for 30

mins. After 30 mins, the animals were transferred to 20°C and the number of eggs laid were counted every hour.

Microscopy

Expression patterns. Images were acquired using a Zeiss Axioskop 2 microscope system and LSM Pascal 5 imaging software (Zeiss). Images were processed using ImageJ software. Worms were mounted on agarose pads and immobilized with 0.3 M sodium azide. All images were obtained from staged young adult animals (~24hrs after the L4 stage).

HSN imaging and quantification. Images were acquired using an Olympus BX51WI spinning disc confocal microscope. Age-matched adults (30 hrs after the L4 stage) were immobilized using 0.3 M sodium azide on slides with 2% agarose pads. z-stack images of HSN synapses and cell bodies were acquired from all animals. Images were analyzed using Volocity6.3 software. Total SNB-1::GFP volume, number of SNB-1::GFP varicosities and GFP::SYD-2 clusters in the HSN synaptic regions were determined as described in Tanis et al., 2008. Mean SNB-1::GFP and SYD-2::GFP intensities at HSN synapses were determined by averaging the mean GFP fluorescence intensities from all SNB-1::GFP and SYD-2::GFP varicosities in a single HSN process (within 10 μm of the vulval slit) and subtracting the threshold value (1000 units or 1500 units above mean background fluorescence for SNB-1::GFP or SYD-2::GFP respectively). DsRed2 intensities were measured similarly in the same exact regions occupied by SNB-1::GFP.

SNB-1::GFP and DsRed2 intensities in HSN cell bodies were determined by calculating their mean intensities within each cell body and subtracting the threshold value

(1500 units above background).

Acknowledgements

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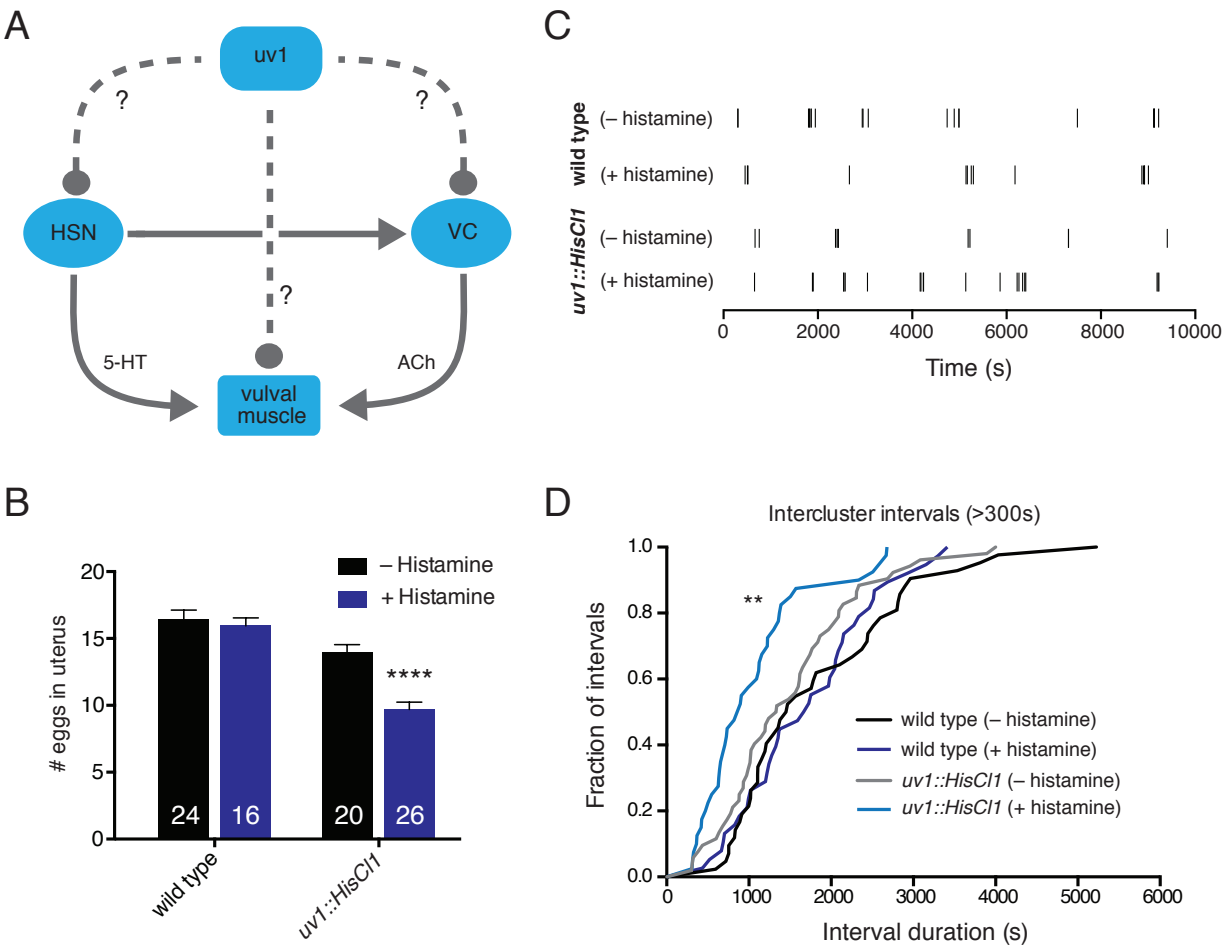


Figure II-1. Reducing the activity of neurosecretory uv1 cells increases egg-laying

(A) Schematic diagram of the egg-laying circuit**.** The HSNs make primarily serotonergic synaptic connections onto Vm2 vulval muscles and the VC5 motor neurons. Cholinergic VC4 and VC5 motor neurons make synaptic connections onto the vulval muscles. Neurosecretory uv1 cells express several neuromodulators but do not have direct postsynaptic targets. Circles represent putative inhibitory connections while arrows denote excitatory connections. Solid lines depict known synaptic connections while dashed lines depict putative extrasynaptic signaling. Connections based on (Schafer,

2006, White et al., 1986, Zhang et al., 2008, Collins and Koelle, 2013). (B) Quantification of eggs *in utero* for wild type and transgenic animals (*ufIs160*) stably expressing the histamine-gated chloride channel in uv1 cells (*uv1::HisCl1*) under control conditions or immediately following exposure (6 hrs) to exogenous histamine (50 mM). *uv1::HisCl1* refers to expression of the *Pocr-2::HisCl1::SL2::GFP::ocr-2* 3' UTR transgene. Bars represent mean ± SEM for each condition. Numbers in bars indicate n for each condition. ****p<0.0001, ANOVA with Sidak's test. (C) Temporal analysis of egg-laying behavior with uv1 silencing. Histamine exposure began 1 hr prior to the start of analysis and continued throughout. Tick marks indicate single egg-laying events. Representative data from one animal is shown for each condition. (D) Cumulative distribution of intercluster intervals between egg-laying events. The frequency of all intervals >300 s is plotted for the conditions indicated. Histamine treatment (light blue) produces a significant leftward shift toward shorter intercluster intervals. WT (– his): n=42; WT (+ his): n=38; *uv1::HisCl1* (-His): n=52, *uv1::HisCl1* (+His): n=40. **p<0.01, Mann-Whitney test. n=number of intervals.

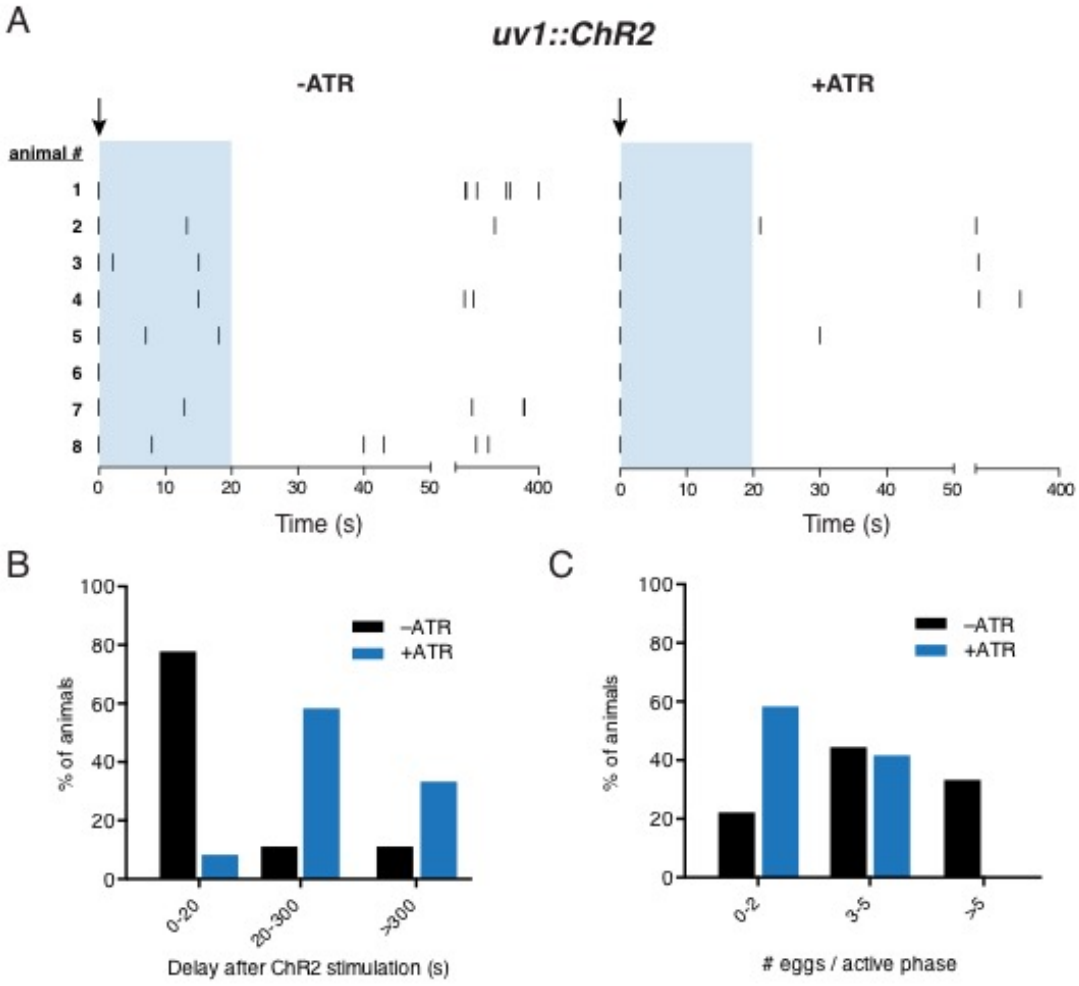


Figure II-2. Acute stimulation of uv1 cells inhibits egg-laying activity

(A) Temporal analysis of egg-laying behavior for 400 s immediately following photostimulation of animals stably expressing ChR2 in the uv1 cells (*vsIs189*; *Pocr-2::ChR2::YFP::ocr-2* 3'UTR) under control conditions (-ATR) or with exposure to exogenous retinal (+ATR). Light stimulation (indicated by arrow) (5 s, 100 W/m^2) was initiated immediately after the first egg-laying event of an active phase. Tick marks indicate single egg-laying events. Representative data from 8 animals for each condition

is shown. Blue shading indicates the 0-20 s time interval quantified in Figure 2B (0-20). Two time periods are shown: (1) 50 s immediately following the first egg-laying event (0-50) and (2) the subsequent 350 s (50-400) on a condensed scale. (B) Quantification of time interval between the onset of light stimulus and subsequent egg-laying event. Percent of animals responding within 20 s, 20-300 s, or after >300 s is shown as indicated. -ATR: n=9, +ATR: n=12, $p<0.0001$, Chi-square test. (C) Quantification of total egg-laying events following the initial event within an active phase. Percent of animals laying 0-2, 3-5, or >5 eggs is shown as indicated. -ATR: n=9, +ATR: n=12, $p<0.0001$, Chi-square test.

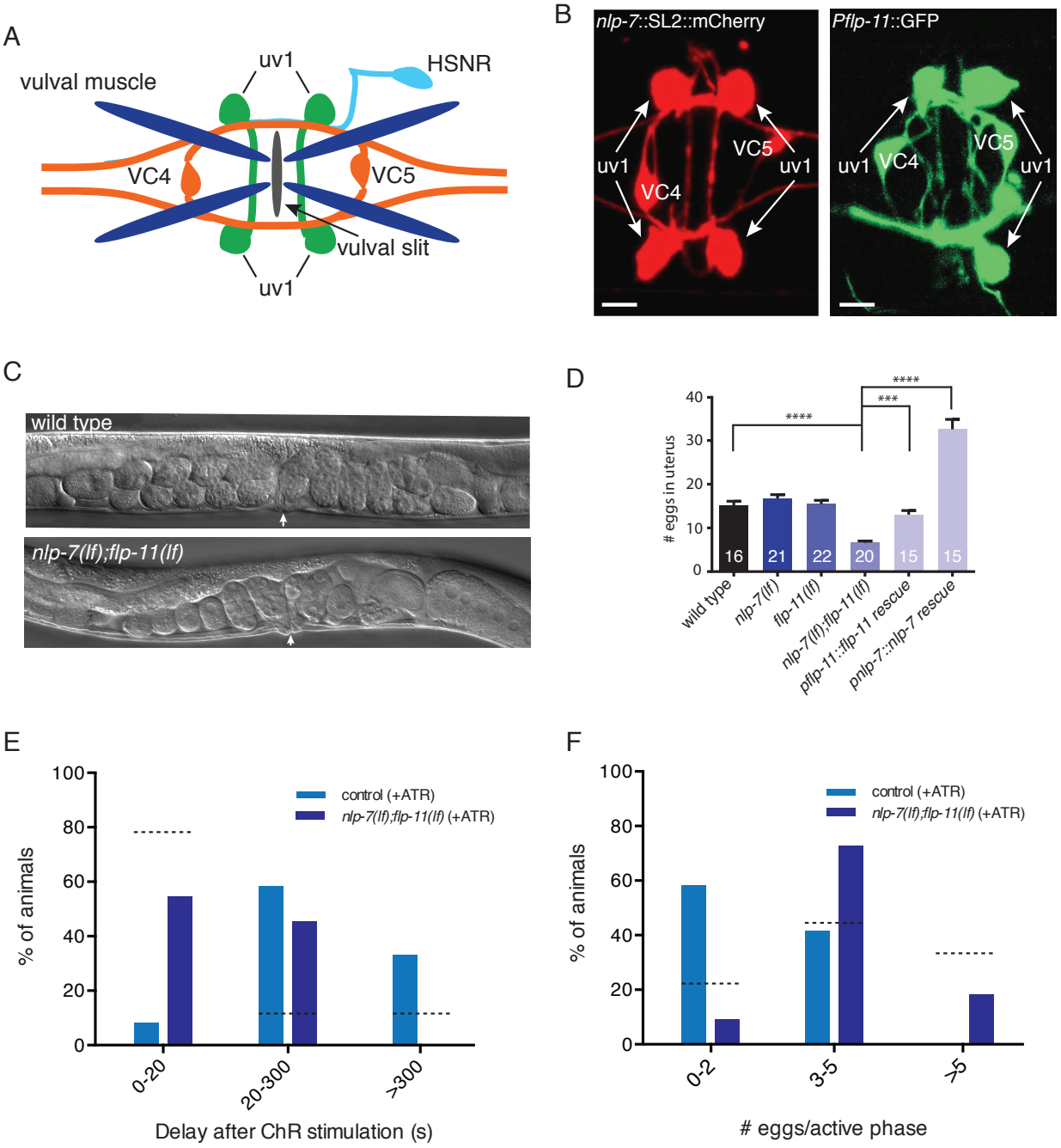


Figure II-3. NLP-7 and FLP-11 are expressed in uv1 cells and act synergistically to inhibit egg-laying

(A) Schematic of egg-laying neuromusculature. (B) Fluorescent images of adult hermaphrodites expressing either *Pnlp-7*::NLP-7::SL2::mCherry or *Pflp-11*::GFP reporter

transgenes. In addition to the egg-laying circuit, both reporters label head and tail neurons (see Figures S1 and S2). Scale bars, 5 μm. (C) Representative DIC images of wild type (upper) and *nlp-7(lf);flp-11(lf)* double mutants (lower). Arrows indicate position of the vulva. (D) Quantification of eggs *in utero*. Bars represent mean ± SEM for each genotype. Numbers in bars indicate the n for each group. **** p<0.0001, *** p<0.001 ANOVA with Sidak's post-hoc test. In this and all subsequent figures, *nlp-7(lf)* and *flp-11(lf)* refer to the *tm2984* and *tm2709* alleles respectively. (E) Time interval between light stimulus and subsequent egg-laying event. For E and F, light stimulation (5 s, 100 W/m^2) of animals stably expressing *uv1::ChR2* was initiated immediately after the first egg-laying event of an active phase. Percent of animals that perform a second egg laying event within 20 s, 20-300 s, or after >300 s is indicated. control, n=12; *nlp-7;flp-11*, n=11. p<0.0001, Chi-square test. Dashed lines indicate percent of animals in each category for control stimulation in the absence of retinal (from Figure 2). (F) Quantification of total egg-laying events following the initial event within an active phase. Percent of animals laying 0-2, 3-5, or >5 eggs following uv1 photostimulation is shown. Dashed lines indicated percent of animals in each category for control stimulation in the absence of retinal (from Figure 2). ATR, exogenous retinal. control, n=12; *nlp-7;flp-11*, n=11. p<0.0001, Chi-square test.

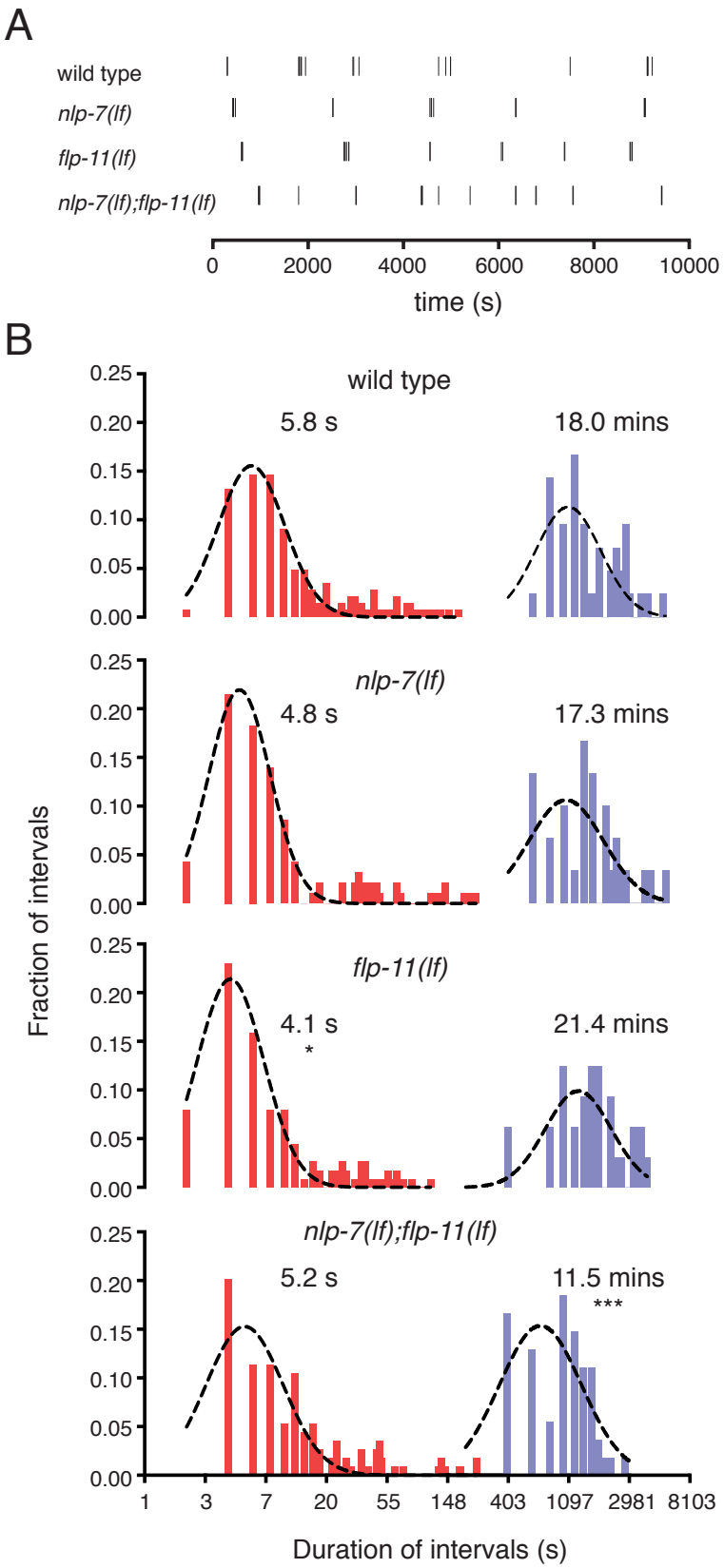

A
wild type
nlp-7(lf)
flp-11(lf)
nlp-7(lf);flp-11(lf)
0
2000
4000
6000
8000
10000
time (s)
B
wild type
5.8 s
18.0 mins
nlp-7(lf)
4.8 s
17.3 mins
flp-11(lf)
4.1 s
*
21.4 mins
nlp-7(lf);flp-11(lf)
5.2 s
11.5 mins
***
Fraction of intervals
0.25
0.20
0.15
0.10
0.05
0.00
1
3
7
20
55
148
403
1097
2981
8103
Duration of intervals (s)

Figure II-4. The duration of inactive egg-laying periods is reduced with combined deletion of *nlp-7* and *flp-11*

(A) Temporal analysis of egg-laying behavior. Tick marks indicate single egg-laying events. Representative data from one animal of each genotype is shown. (B) Histograms of intervals between egg-laying events. Intervals are plotted on a natural log scale on the x-axis and their relative frequencies are plotted on the y-axis. Red indicates intracluster intervals (<300 s) and blue indicates intercluster intervals (>300 s). Dashed lines indicate curve fit to Gaussian distribution. Mean intracluster and intercluster intervals for each genotype were calculated from the curve fit (wild type: 5.8 ± 1 s and 18.0 ± 0.02 mins; *nlp-7(lf)*: 4.8 ± 1 s and 17.3 ± 0.02 mins; *flp-11(lf)*: 4.1 ± 1 s and 21.4 ± 0.02 mins; *nlp-7(lf);flp-11(lf)*: 5.2 ± 1 s and 11.5 ± 0.02 mins). wild type: n1=144, n2=42; *nlp-7(lf)*: n1=93, n2=30; *flp-11(lf)*: n1=113, n2=32; *nlp-7(lf);flp-11(lf)*: n1=114, n2=54. n1 = number of intracluster intervals (<300 s), n2 = number of intercluster intervals (>300 s). ***p<0.001, *p<0.05, Kruskal Wallis test with Dunn's multiple comparisons test. The average intercluster interval (inactive phase) is significantly shortened in *nlp-7;flp-11* double mutants and egg-laying occurs at earlier developmental stages compared with wild type. These effects are rescued by uv1-specific expression of either *nlp-7* or *flp-11* (see Figure S3).

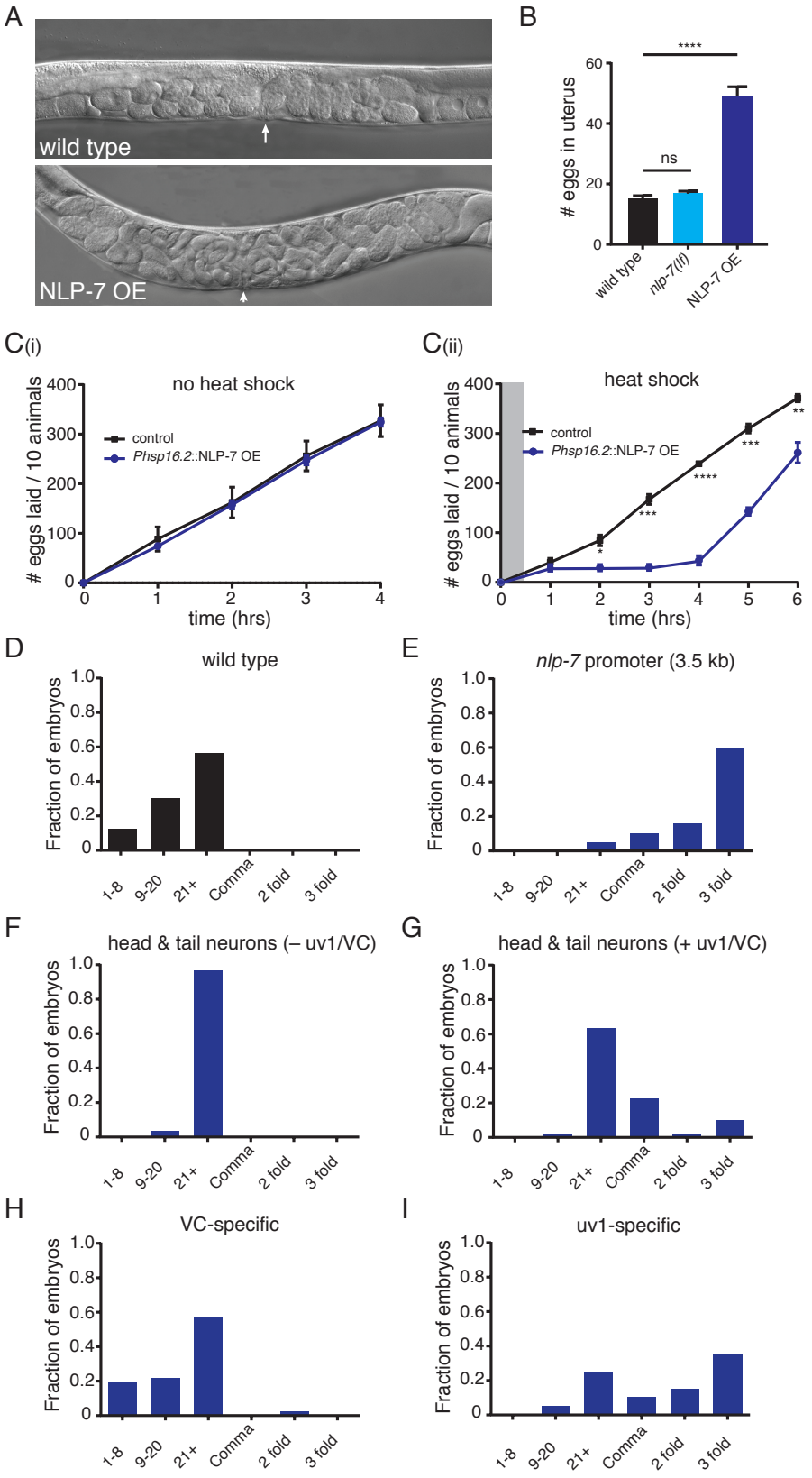

A
wild type
NLP-7 OE
B
eggs in uterus
****
ns
wild type
nlp-7(lf)
NLP-7 OE
C(i)
no heat shock
eggs laid / 10 animals
control
Phsp16.2::NLP-7 OE
time (hrs)
C(ii)
heat shock
D
wild type
Fraction of embryos
1-8
9-20
21+
Comma
2 fold
3 fold
E
nlp-7 promoter (3.5 kb)
F
head & tail neurons (– uv1/VC)
G
head & tail neurons (+ uv1/VC)
H
VC-specific
I
uv1-specific

Figure II-5. NLP-7 expression in the uv1 cells is required for the inhibition of egg-laying

(A) Representative DIC images of wild type (upper) and transgenic animals (*uflS118)* stably expressing high levels of *nlp-7* genomic sequence (NLP-7 OE, lower). Arrows indicate position of the vulva. (B) Quantification of eggs *in utero*. Bars represent the mean ± SEM for each genotype. n≥20 for all measurements. ****p<0.0001, ANOVA with Sidak's post hoc test. (C) Cumulative number of eggs laid over time in transgenic animals expressing the *nlp-7* genomic sequence under control of a heat shock promoter (*Phsp16.2::*NLP-7 OE) or control animals (non-transgenic siblings). Animals were either (i) maintained continuously at 20°C or (ii) exposed to heat shock (33°C, 30 mins). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, t-test with Holm-Sidak's correction for multiple comparisons. Gray shading indicates the timing and duration of heat shock. (D-I) Distribution of the developmental stages of eggs laid by either wild type adult hermaphrodites (D), or animals overexpressing *nlp-7* from a 3.5 kb *nlp-7* promoter (E), a 2.5 kb *nlp-7* promoter in which uv1 and VC fluorescence is not observed (F), a 2.5 kb *nlp-7* promoter in which uv1 and VC fluorescence is observed (G), a VC neuron specific promoter (*Plin-11::pes-10*) (H), or a uv1 promoter (*ocr-2*) (I). Animals overexpressing NLP-7 from the 3.5 kb promoter (E) lay a significantly higher fraction of late-stage embryos compared with wild type (p<0.0001, Fisher's exact test). Exclusive expression in head and tail neurons produces no significant change (F), while there is a significant shift to later developmental stages when fluorescence is observed near the vulva (G) (p<0.0001, Fisher's exact test). uv1-specific expression also produces a significant shift (p<0.0001, Fisher's exact test) while VC-specific expression produces no significant

difference. For each genotype, eggs laid within a 30 minute time period were evaluated. See also Figure S3.

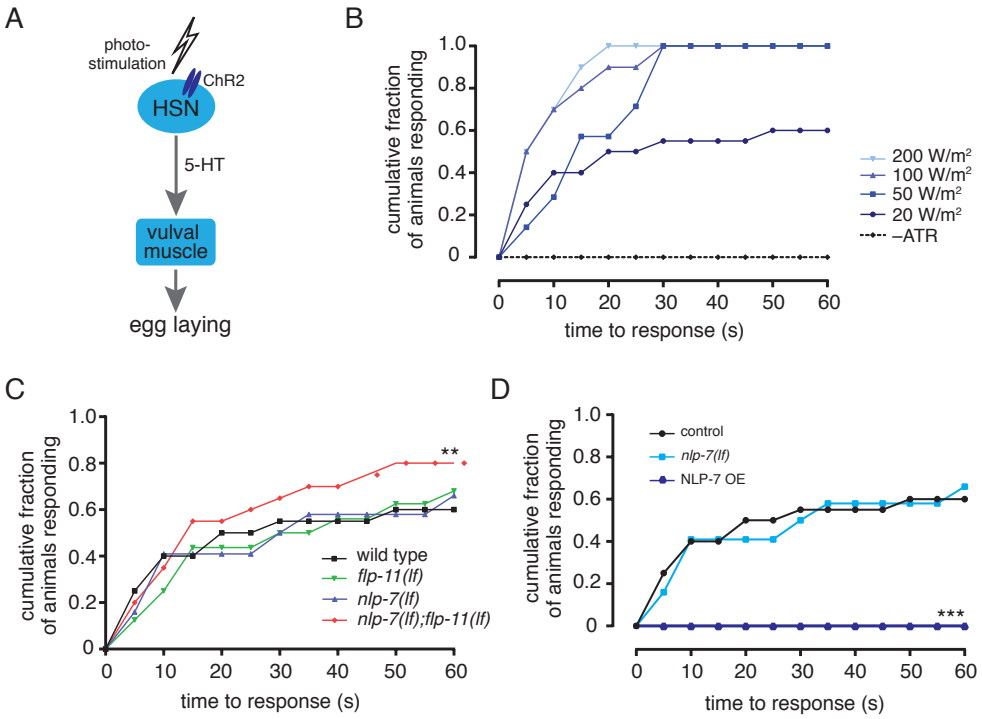


Figure II-6. NLP-7 and FLP-11 regulate the activity of the egg-laying circuit.

(A) Schematic diagram depicting optogenetic stimulation of the HSN neurons. Photostimulation of channelrhodopsin expressed in the HSNs (*Pegl-6a*::ChR2::YFP) elicits bouts of egg-laying (Emtage et al., 2012)(Movie S3). (B) Cumulative fraction of animals that respond to blue light exposure over time. Latency to initial egg-laying event following light stimulus for varying light intensities. n=10 for each condition. –ATR refers to control stimulation (50 W/m^2) of the same genotype in the absence of retinal. (C) Cumulative plot of latency to initial egg-laying event following light stimulus (20 W/m^2). Wild type, n=20; *flp-11(lf)*, n=16; *nlp-7(lf)*, n=14; *nlp-7(lf);flp-11(lf)*, n=20. **p<0.01, Wilcoxon matched-pairs test. (D) Cumulative plot of latency to initial egg-laying event following light stimulus (20 W/m^2). ***p<0.001, Wilcoxon matched-pairs test.

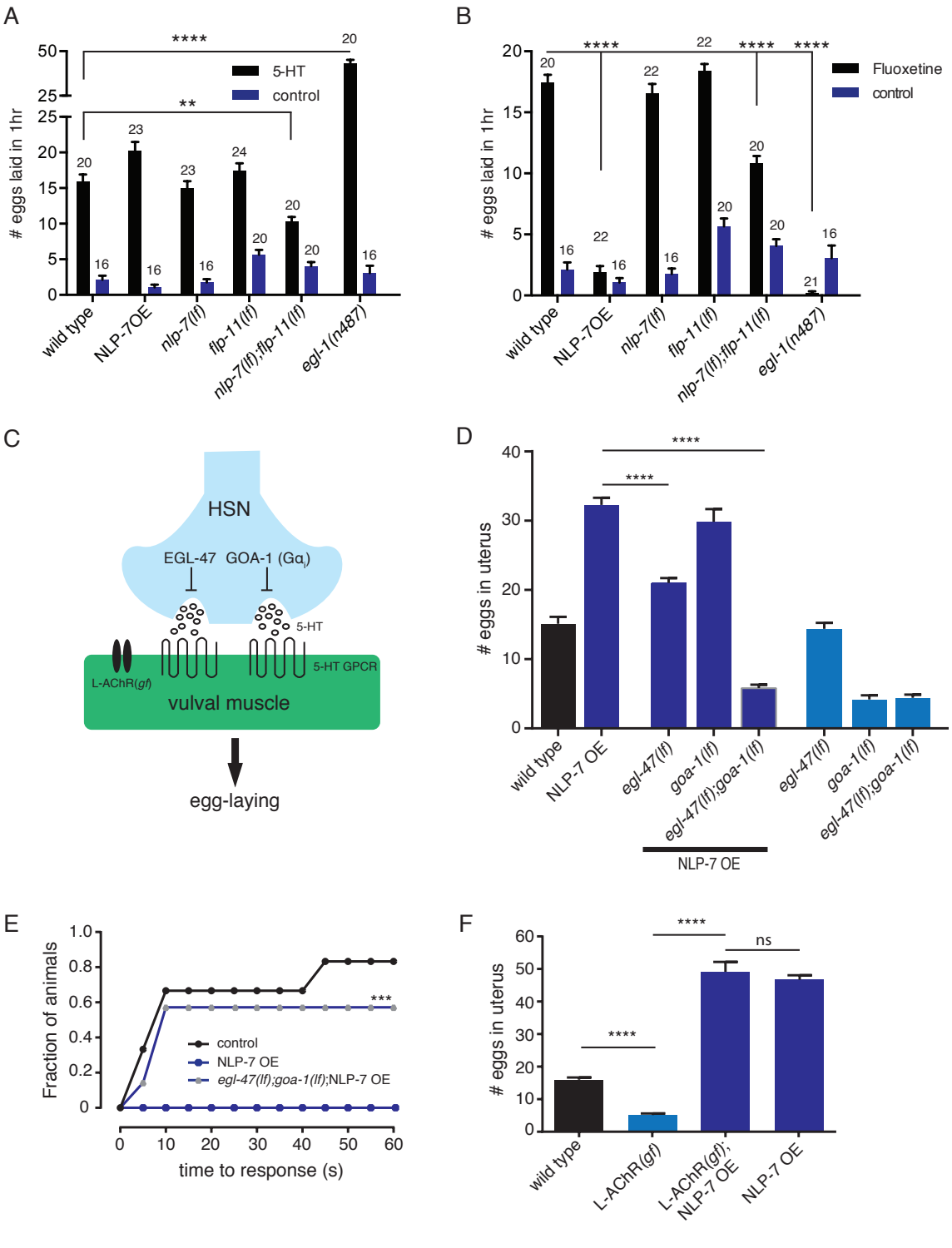

A
eggs laid in 1hr
5-HT
control
****
**
wild type
NLP-7OE
nlp-7(lf)
flp-11(lf)
nlp-7(lf);flp-11(lf)
egl-1(n487)
B
eggs laid in 1hr
Fluoxetine
control
****
wild type
NLP-7OE
nlp-7(lf)
flp-11(lf)
nlp-7(lf);flp-11(lf)
egl-1(n487)
C
HSN
EGL-47
GOA-1 (Gαo)
5-HT
5-HT GPCR
L-AChR(gf)
vulval muscle
egg-laying
D
eggs in uterus
****
wild type
NLP-7 OE
egl-47(lf)
goa-1(lf)
egl-47(lf);goa-1(lf)
NLP-7 OE
E
Fraction of animals
time to response (s)
***
control
NLP-7 OE
egl-47(lf);goa-1(lf);NLP-7 OE
F
eggs in uterus
****
ns
wild type
L-AChR(gf)
L-AChR(gf); NLP-7 OE
NLP-7 OE

Figure II-7. NLP-7 reduces the activity of the HSNs

(A) Quantification of eggs laid in 1 hour for the genotypes indicated, in either control buffer or in the presence of exogenous serotonin (7.5 mg/ml). Bars represent mean ± SEM for each condition. Numbers above bars indicate n for each condition. ****p<0.0001 ANOVA with Sidak's post hoc test. For A and B, *egl-1* mutants in which the HSNs fail to develop are included as a control. 5-HT=serotonin. See also Figure S4 for comparison with egg-laying on plates. (B) Quantification of eggs laid in 1 hour for the genotypes indicated in either control buffer or in the presence of the serotonin reuptake inhibitor fluoxetine (0.5 mg/ml). Bars represent mean ± SEM for each condition. Numbers above bars indicate n for each condition. **p<0.01, ****p<0.0001 ANOVA with Sidak's post hoc test. (C) Schematic of genetic manipulations used to modify egg-laying activity. See text for details. (D) Quantification of eggs *in utero*. Bars represent the mean ± SEM for each genotype. n≥20 for all measurements. ****p<0.0001, ANOVA with Sidak's post hoc test. (E) Cumulative plot of latency to initial egg-laying event following light stimulus (50 W/m^2) the genotypes indicated expressing *Pegl-6a*::ChR2::YFP. n≥15 for all measurements. ***p<0.001, Wilcoxon matched pair test. (F) Quantification of eggs retained *in utero*. Bars represent mean ± SEM for each genotype. L-AChR*(gf)* refers to stable muscle-specific expression of AChRs with elevated ACh responsiveness (Bhattacharya et al., 2014). n≥15, ****p<0.0001, ANOVA with Sidak's post hoc test.

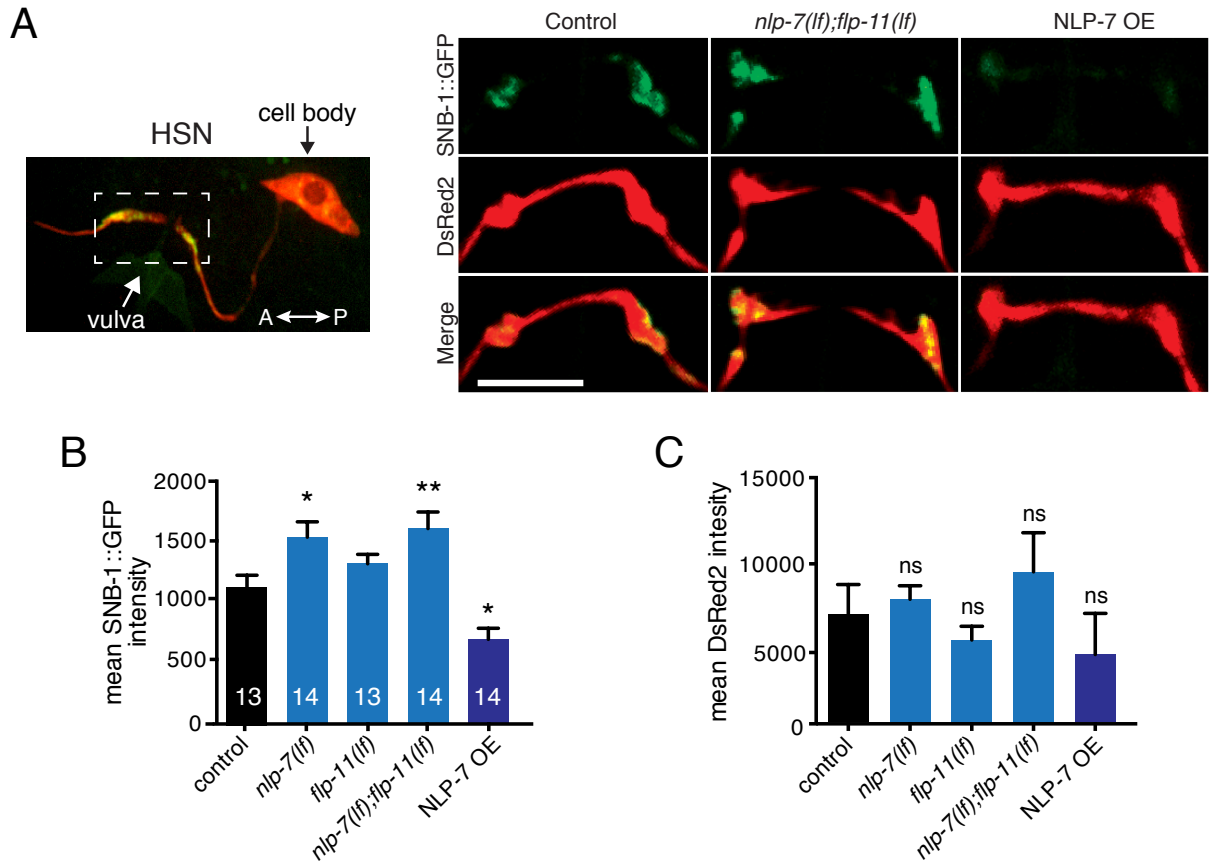
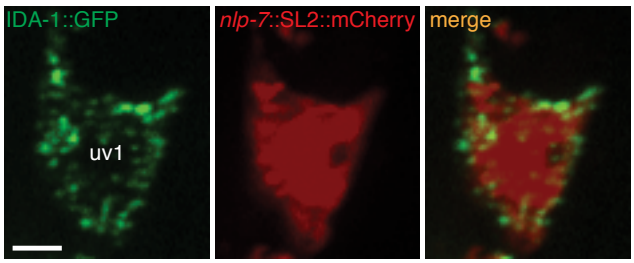


Figure II-8. NLP-7 signaling regulates SNB-1::GFP abundance at HSN synapses

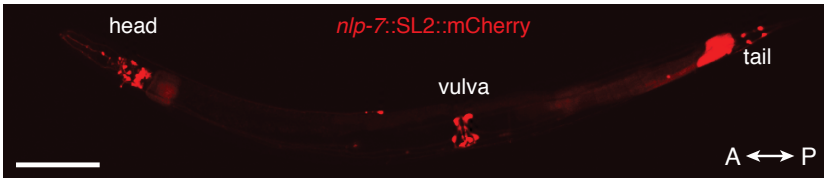
(A) Left, confocal image of HSN cell body and synaptic regions. Dashed white box indicates approximate area of synaptic regions detailed at right and used for analysis. Right, representative confocal images of HSN presynaptic varicosities in transgenic animals expressing the synaptic vesicle marker SNB-1::GFP and DsRed2 in the HSNs (*vsIs103*, *Ptph-1*::SNB-1::GFP; *Ptph-1*::DsRed2) (Tanis et al., 2008) for the genotypes indicated. (B, C) Quantification of average SNB-1::GFP (B) or DsRed2 (C) intensity in synaptic region of the HSNs for the genotypes indicated. Bars represent mean ± SEM for each condition. See also Figures S5 and S6 for additional quantification of SNB-1::GFP and the active zone marker GFP::SYD-2. SNB-1::GFP fluorescent intensity is significantly

increased or decreased in HSN synaptic regions of *nlp-7* mutants or NLP-7 OE animals respectively (see model in Figure S7). Numbers in bars indicate the n for each condition. *p<0.05, **p<0.01, ANOVA with Sidak's post hoc test. Scale bar, 7 μm.

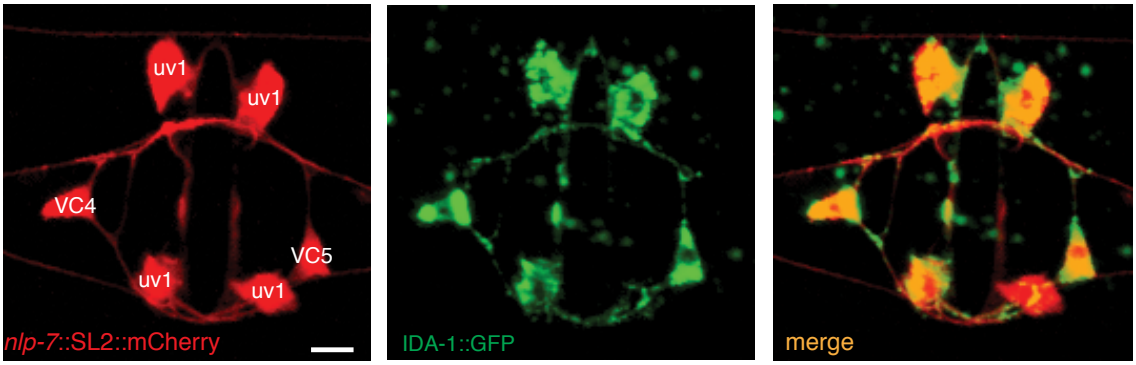
A


IDA-1::GFP
nlp-7::SL2::mCherry
merge
uv1

B


head
nlp-7::SL2::mCherry
tail
vulva
A ⟷ P

C


uv1
uv1
VC4
VC5
uv1
uv1
nlp-7::SL2::mCherry
IDA-1::GFP
merge

D

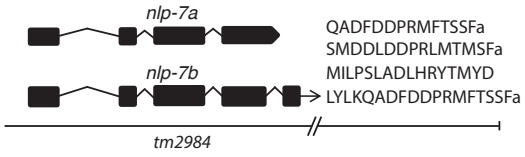
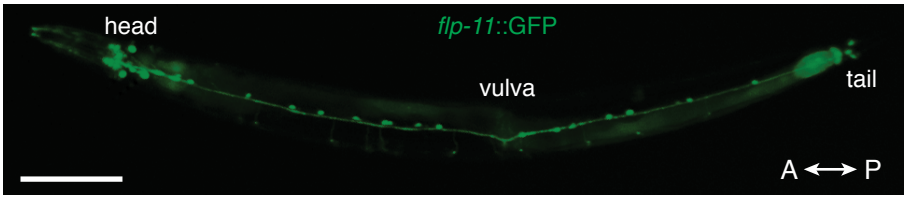

nlp-7a
QADFDDPRMFTSSFa
SMDDLDDPRLMTMSFa
nlp-7b
MILPSLADLHRYTMYD
LYLKQADFDDPRMFTSSFa
tm2984

Figure II-9. *nlp-7* gene structure, mutation and expression

(A) Representative confocal images showing distribution of the dense core vesicle marker IDA-1::GFP (green) in a single uv1 cell. IDA-1::GFP shows a punctate distribution and is enriched near the cell periphery. Scale bar, 2 μm. (B) Fluorescent images of whole animals expressing P*nlp-7::nlp-7*::SL2::mCherry. Scale bar, 100 μm. (C) Representative confocal images showing coexpression of P*nlp-7*::SL2::mCherry with P*ida-1*::IDA-1::GFP in the uv1 cells, VC4 and VC5 motor neurons. Scale bar, 5 μm. (D) *nlp-7* gene structure. Solid boxes represent exons. Sequences deleted in *tm2984* allele are indicated. Predicted peptide products are shown to the right of corresponding gene models.

A



B

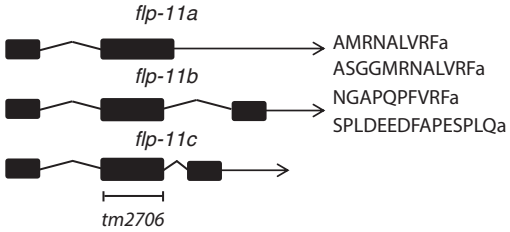


Figure II-10. *flp-11* gene structure, mutation and expression

(A) Fluorescent confocal images of whole animals expressing P*flp-11*::GFP. Scale bar, 100 μm. (B) *flp-11* gene structure. Solid boxes represent exons. Sequences deleted in *tm2706* allele are indicated. Predicted peptide products are shown to the right of corresponding gene models.

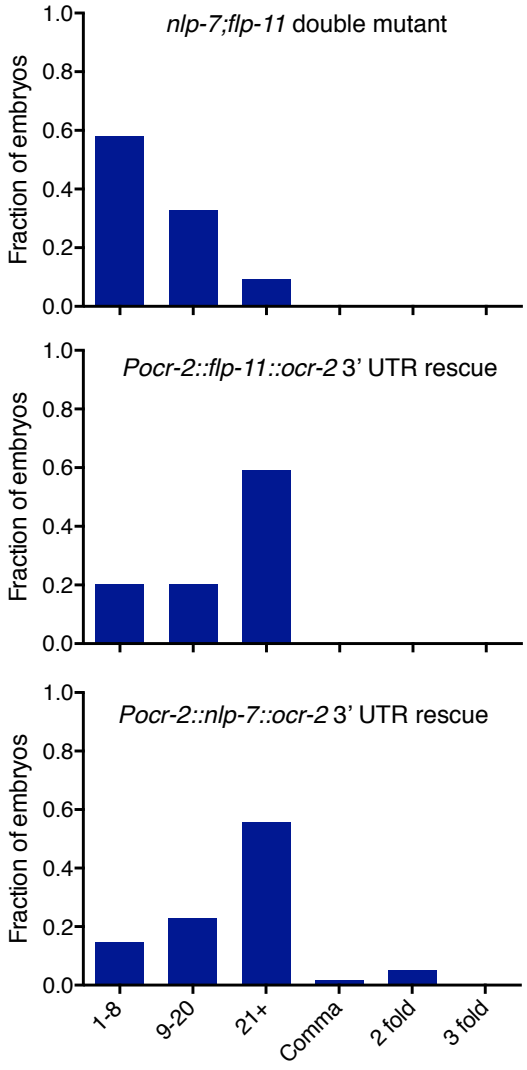


Figure II-11. uv-1-specific expression of *flp-11* reverses constitutive egg-laying in *nlp-7;flp-11* double mutants

Distribution of the developmental stages of eggs laid by either *nlp-7;flp-11* double mutants (upper) or with uv1-specific expression of either *flp-11* (middle) or *nlp-7* (lower) in *nlp-7;flp-11* double mutants. uv1-specific expression of either precursor produced a significant decrease in the proportion of eggs that were laid as 1-8 cell embryos. $p<0.0001$, Fisher's exact test.

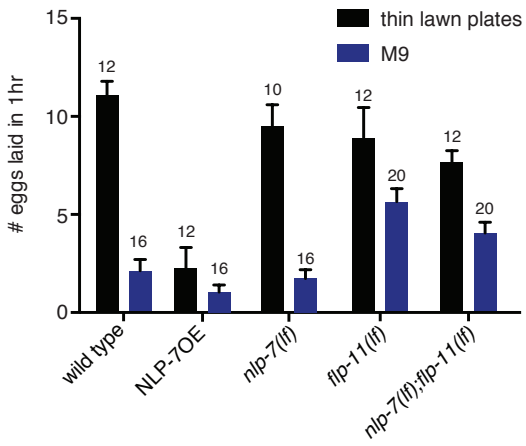


Figure II-12. Comparison of egg-laying rate in M9 versus plates

Quantification of eggs laid in 1 hour for the genotypes indicated, in either control buffer (M9) or on NGM plates seeded with a thin bacterial lawn. Bars represent mean ± SEM for each condition. Numbers above bars indicate n for each condition. Data for M9 were duplicated from Figure 7.

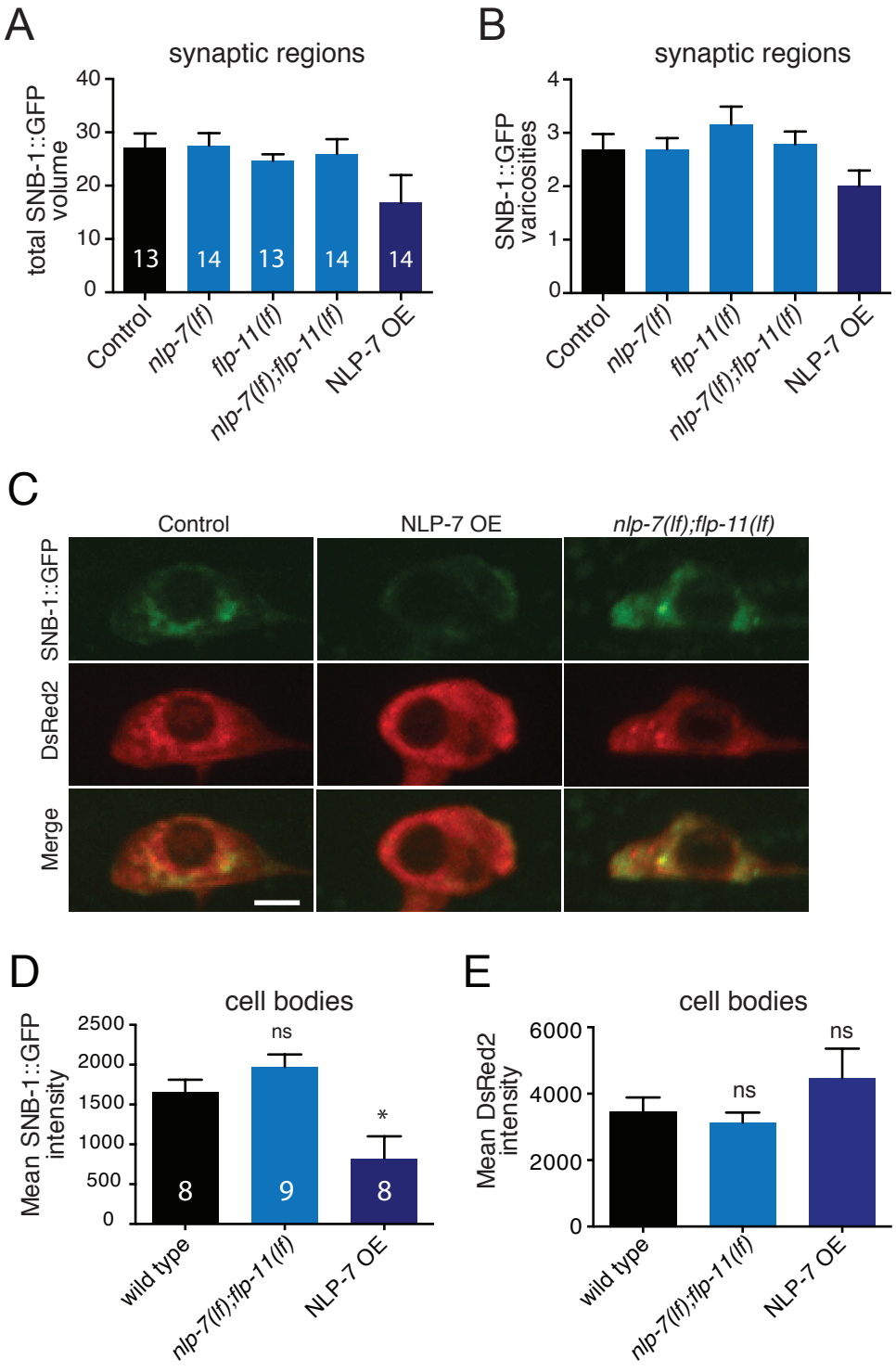

A
synaptic regions
total SNB-1::GFP volume
40
30
20
10
0
13
14
13
14
14
Control
nlp-7(lf)
flp-11(lf)
nlp-7(lf);flp-11(lf)
NLP-7 OE
B
synaptic regions
SNB-1::GFP varicosities
4
3
2
1
0
Control
nlp-7(lf)
flp-11(lf)
nlp-7(lf);flp-11(lf)
NLP-7 OE
C
Control
NLP-7 OE
nlp-7(lf);flp-11(lf)
SNB-1::GFP
DsRed2
Merge
D
cell bodies
Mean SNB-1::GFP intensity
2500
2000
1500
1000
500
0
ns
*
8
9
8
wild type
nlp-7(lf);flp-11(lf)
NLP-7 OE
E
cell bodies
Mean DsRed2 intensity
6000
4000
2000
0
ns
ns
wild type
nlp-7(lf);flp-11(lf)
NLP-7 OE

Figure II-13. Characterization of synapses and cell bodies of HSNs

(A, B) Quantification of total SNB-1::GFP volume (A) and number of SNB-1::GFP varicosities (B) in HSN synaptic regions for the genotypes indicated. SNB-1::GFP volume greater then 1 μm^3 was considered as a varicosity (Tanis et al., 2008). (C) Representative confocal images of HSN cell bodies in transgenic animals expressing the synaptic vesicle marker SNB-1::GFP and DsRed2 in the HSNs (*vsIs103*, *Ptph-1*::SNB-1::GFP; *Ptph-1*::DsRed2) for the genotypes indicated. Scale bar, 3 µm. (D, E) Quantification of average SNB-1::GFP (D) or DsRed2 (E) intensity in cell bodies of the HSNs for the genotypes indicated. Bars represent mean ± SEM for each condition. Numbers in bars indicate the n for each condition. *p<0.05, ANOVA with Sidak's test.

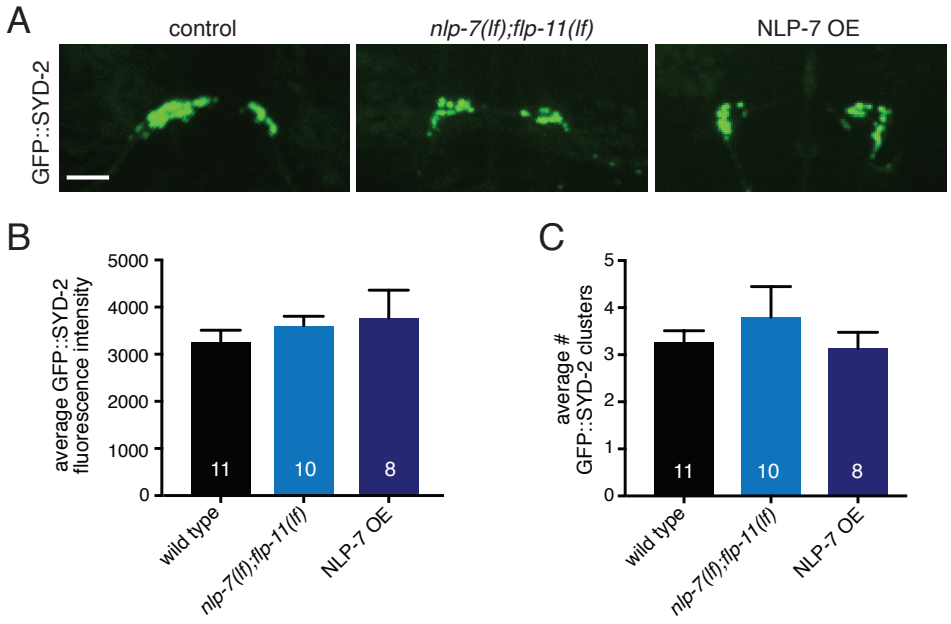


Figure II-14. Localization of active zone marker SYD-2 in HSNs

(A) Representative confocal images of HSN synapses in transgenic animals expressing the active zone marker GFP::SYD-2 in the HSNs (*wyIs12*, *Punc-86*::GFP::SYD-2; *Podr-1*::GFP) for the genotypes indicated. Scale bar, 3 μm. (B, C) Quantification of average GFP::SYD-2 intensity (B) or number of SYD-2 clusters (C) in the HSN synaptic region for the genotypes indicated. Bars represent mean ± SEM for each condition. Numbers in bars indicate the n for each condition.

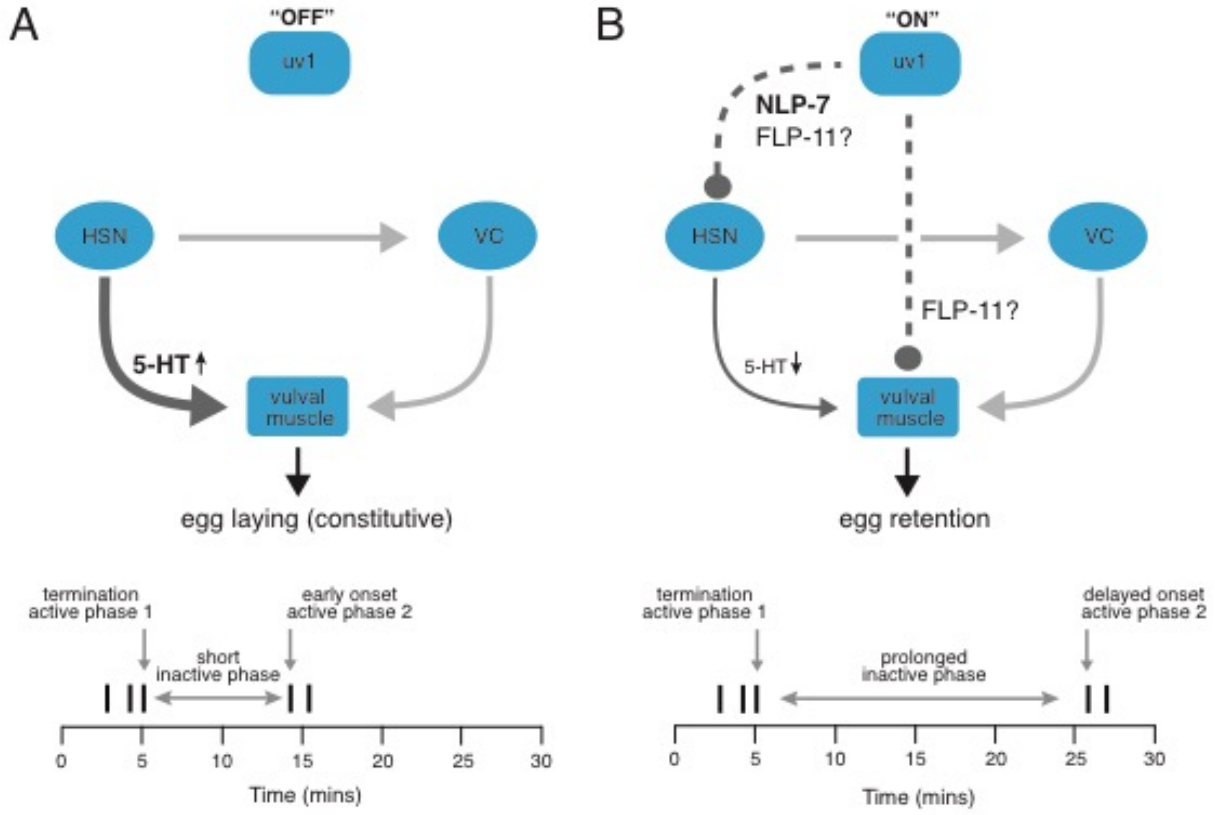


Figure II-15. NLP-7 and FLP-11 shape the timing of egg-laying through negative regulation of serotonergic HSN signaling

(A) During the uv1 “off” phase, spontaneous activity of the HSN neurons elicits serotonin release onto the Vm2 vulval muscles, triggering entry into an active phase of egg-laying. Reduced levels of uv1 activity promote “short” inactive phases (bottom). (B) During the uv1 “on” phase, activation of the uv1 cells triggers release of NLP-7 and FLP-11 peptides. Release of these peptides promotes the termination of an active phase and lengthens the

duration of the inactive phase (bottom), at least in part, by reducing serotonergic activation of vulval muscles. Under normal (favorable) conditions, cycles of uv1 activity shape the timing of egg-laying events. Solid lines indicate synaptic connections. Dashed lines indicate volume transmission. Arrows indicate excitation. Circles indicate inhibition. In the lower panel of A and B, each tick mark represents a single egg-laying event.

Movie II-1. Egg-laying response to uv1 photostimulation

Light stimulation of the uv1 cells inhibits egg-laying in a control animal expressing *Pocr-2::ChR2::YFP::ocr-2* 3'UTR. Egg-laying events are indicated by numbers. Light stimulation is initiated immediately following the first egg-laying event. The timing and duration of blue light are indicated by blue text. The animal performs an avoidance response upon blue light exposure, likely due to ChR2 expression in 2-3 head sensory neurons (see Methods). Video is played 6X faster than real time.

Movie II-2. Egg-laying response to uv1 photostimulation

nlp-7(lf);flp-11(lf) double mutant animal expressing channelrhodopsin in the uv1 cells continues egg-laying following light stimulation of the uv1 cells. Note that the avoidance response to light stimulation is unaffected by combined deletion of *nlp-7* and *flp-11*, indicating that NLP-7 and FLP-11 peptides are required for inhibition of the egg-laying response but not for the execution of the avoidance behavior. Egg-laying events are indicated by numbers (black). Light stimulation is initiated immediately following the first egg-laying event. The timing and duration of stimulation are indicated by blue text. Video is played 6X faster than real time.

Movie II-3. Egg-laying response to HSN photostimulation

Light stimulation of the HSNs in a control animal expressing *Pegl-6a*::ChR2::YFP. Timing of light stimulation is indicated by white text. The animal initiates a burst of egg-laying a few seconds following the onset of light stimulation.

Preface for Chapter III

The work presented in this chapter elucidates the downstream signaling machinery involved in neuropeptide mediated inhibition of egg-laying behavior in *C. elegans*. The results shown in chapter II had demonstrated a role for the neuropeptides NLP-7 and FLP-11 in regulating serotonergic transmission to control the duration of distinct states in the egg-laying behavioral program. This chapter is an attempt to decode the molecular signaling candidates that mediate the actions of the two neuropeptides. A candidate genetic suppressor screen has identified some of the molecular signals that may act as receptors for the neuropeptides. In addition, a EMS mutagenesis screen has also identified a suppressor allele that may be one of the downstream effectors involved in the inhibitory effects of the neuropeptides.

Navonil Banerjee designed and performed the experiments and analyzed the data. Navonil Banerjee generated transgenic strains. The work presented in this chapter is a work in progress at the time of thesis preparation.

Chapter III: Decoding downstream signaling mechanisms for neuropeptide regulation of *C. elegans* egg-laying behavior

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Abstract

Neuropeptide signaling play critical roles in establishing stable behavioral states and coordinating transitions between them. However, identifying the molecular candidates that mediate the actions of neuropeptides *in vivo* has largely remained a challenge. By studying neuropeptidergic signaling in the context of *C. elegans* egg-laying behavior, we have made an attempt to elucidate downstream molecular candidates mediating the inhibitory effects of the neuropeptides NLP-7 and FLP-11 on egg-laying activity. We have performed a candidate genetic suppressor screen and have identified the GPCRs encoded by the genes *npr-7, npr-8 and frpr-3,* that might be acting as potential receptors for NLP-7 and FLP-11 in mediating their modulatory control on the egg-laying circuit. In addition, we have also performed a genome-wide EMS mutagenesis screen and have identified a strong suppressor of NLP-7 mediated inhibition of egg-laying. Future studies aimed at detailed characterization of the receptors as well as the suppressor would provide useful insights into *in vivo* mechanisms governing neuropeptide signaling that refines activity within the egg-laying circuit to shape the temporal pattern of egg-laying behavior.

Introduction

Neuropeptide signaling has been implicated in the temporal control of several behavioral programs. However, elucidating the downstream signaling mechanisms by which neuropeptides exert their effects on neural circuits remains a challenge. A critical step in elucidating mechanisms mediating the actions of neuropeptides is identifying their respective receptors. G-protein coupled receptors (GPCRs) have been largely attributed for mediating neuropeptidergic signaling. GPCRs have slower kinetics of action and are therefore more suitable for generating stable and long-lasting behavioral states.

The functional characterization of neuropeptide GPCRs has been expedited by the use of *C. elegans* as a model system. Availability of genetic mutants as well as ease of generating reporter transgenics have shed light on the respective functions and expression patterns of several candidate GPCRs. Of all the protein coding genes in the *C. elegans* genome, around 7% encode GPCRs (Bargmann, 1998; Fredriksson and Schioth, 2005). GPCRs in C. elegans have been shown to mediate several behavioral outcomes ranging from locomotion or reproduction to social behavior.

GPCR signaling mechanisms in *C. elegans* are remarkably conserved with that in mammals. In the classical GPCR signaling pathway, the inactive receptor is bound to the $G_{\alpha\beta\gamma}$ heterotrimeric protein. Upon activation by its corresponding ligand, the $G_{\alpha\beta\gamma}$ dissociates into a GTP bound G_{α} (G_{α}-GTP) and a $G_{\beta\gamma}$ subunit. G_{α}-GTP activates different effectors such as phospholipase Cβ (PLCβ) or adenylyl cyclase depending on the G_{α} subtype. The $G_{\beta\gamma}$ subunit also regulates certain downstream effector proteins such as ion channels or PLCβ (Figure III-1).

However, lack of sequence similarity of neuropeptide GPCRs in *C. elegans* with vertebrate or insect GPCRs have limited the identification of candidate GPCRs responsible for neuropeptidergic signaling in *C. elegans.* A few approaches have been successful in matching GPCRs with their activating neuropeptide ligands. One approach had taken advantage of reverse pharmacology – expressing candidate GPCRs in heterologous systems and screening for activation with libraries of ligands (Mertens et al., 2004; Beets et al., 2011). Another approach in predicting neuropeptide GPCRs is to use all deorphanized GPCRs as a seeding set in a Multiple Expectation Maximization for Motif Elicitation/Motif Alignment and Search Tool (MEME/MAST) analysis (Jannsen et al., 2010; Frooninckx et al., 2012). Using this approach, a list of 99 genes encoding 125 putative neuropeptide GPCRs have been identified and 23 GPCRs have been deorphanized till date.

We had previously identified two neuropeptides encoded by the genes *nlp-7* and *flp-11* that are involved in regulating the temporal organization of *C. elegans* egg-laying behavior. Here, we make an attempt to identify the receptor(s) for these neuropeptides and elucidate the underlying downstream signaling machinery responsible for mediating their actions.

Results

A candidate genetic screen to identify receptors mediating NLP-7 and FLP-11 inhibition of egg-laying.

In Chapter II, we had shown that local neuromodulation by a group of neurosecretory cells (uv1) direct the timing of egg-laying events. These effects are primarily mediated by neuropeptides encoded by the genes – *nlp-7* and *flp-11*, that inhibit serotonin release from the HSN motorneurons and prolong the duration of inactive phases in the egg-laying behavioral program. To understand how the behavioral effects of these neuropeptides are encoded at a molecular level, we have performed a candidate genetic suppressor screen to identify potential GPCRs that act downstream of these neuropeptides.

Based on our previous results, we have found that overexpression of NLP-7 (NLP-7 OE) leads to a chronic inhibition of egg-laying and over time, leads to a severe egg-laying defect. Here, we use this strong egg-laying defective phenotype of NLP-7 OE animals as a tool to unravel downstream molecular pathways mediating its action. We had demonstrated that the egg-laying defect of NLP-7 OE animals was partially suppressed by a loss-of-function mutation in *egl-47,* which encodes a transmembrane protein bearing homology to the *Drosophila* gustatory receptor family of proteins (Bargmann, 2006). Although EGL-47 was previously thought to be a GPCR (Moresco and Koelle, 2004), later studies had predicted that it displays an inverted membrane topology compared with conventional GPCRs (Bargmann, 2006) and may function as a chloride channel (Tanis et al., 2009). We searched for candidate GPCR loss-of-function mutants

that suppress the residual egg-laying defective phenotype of *egl-47(lf)*;NLP-7 OE animals. This approach was further supported by our previous findings that a loss-of-function mutation in *goa-1,* the *C. elegans* ortholog of the mammalian G_{ai} subunit, completely suppresses the residual egg-laying defect of *egl-47(lf)*;NLP-7 OE animals. Our working hypothesis was that the GPCR(s) which could act as potential receptor(s) for *nlp-7* encoded peptides, would most likely be associated with the inhibitory G-protein subunit GOA-1. Consequently, that GPCR would function in the same genetic pathway as GOA-1, and would presumably lead to complete suppression of the egg-laying defect of *egl-47(lf)*;NLP-7 OE animals.

A previous study had listed 99 genes encoding GPCRs that may act as putative neuropeptide receptors (Jannsen et al., 2010). We selected our candidates from that list of GPCRs and crossed loss-of-function GPCR mutants into the *egl-47(lf)*;NLP-7 OE strain. To look for candidate suppressors, we quantified the number of eggs *in utero*. We found that loss-of-function mutations in *npr-7* and *npr-8* partially suppressed the residual egg-laying defect of *egl-47(lf)*;NLP-7 OE animals. Both *npr-7(lf);egl-47(lf)*;NLP-7 OE and *npr-8(lf);egl-46(lf)*;NLP-7 OE transgenic animals retained a number of eggs *in utero* that was intermediate between wild type and *egl-47(lf)*;NLP-7 OE animals. While wild type animals typically retained 15 eggs on average and *egl-47(lf)*;NLP-7 OE animals retained 29 ± 1 eggs *in utero* under favorable conditions, *npr-7(lf);egl-47(lf)*;NLP-7 OE and *npr-8(lf);egl-47(lf)*;NLP-7 OE retained about 21 and 22 eggs respectively (Figure III-2). These results indicate that NPR-7 and NPR-8 could act as potential receptors for NLP-7 mediated inhibition of egg-laying. Both of these receptors fall under the category of rhodopsin family of GPCRs and show resemblance to neuropeptide Y-like GPCRs in

insects and mammals (Frooninckx et al., 2012). NPR-7 had been previously shown to play a role in fat metabolism and locomotion in *C. elegans* (Keating et al., 2003; Cohen et al., 2009) but any roles of NPR-7 and NPR-8 with regard to egg-laying behavior has not been demonstrated. Both *npr-7(lf)* and *npr-8(lf)* mutants do not show appreciable changes in the number of eggs retained *in utero* (Figure III-2) which suggests that they may act redundantly with each other or some other protein. Moreover, this does not rule out the possibility that they may play roles in other aspects of reproductive behavior such as regulating brood size. The cognate ligands for either receptor has not been elucidated yet. It remains to be seen whether peptide encoded by *nlp-7* could activate NPR-7 and/or NPR-8 in a heterologous system. Further, it would be interesting to know where do these receptors function to mediate NLP-7 inhibition of egg-laying. A crucial step would be to determine the expression patterns of these receptors and performing cell-specific rescue experiments to elucidate their sites of action.

The GPCR FRPR-3 may be involved in FLP-11 mediated inhibition of egg-laying

We have not been able to adopt a similar approach to identify potential receptors involved in FLP-11 mediated inhibition of egg-laying because unlike NLP-7 OE, overexpression of FLP-11 did not lead to a strong egg-laying defective phenotype. However, in the candidate screen described above, we found that loss-of-function mutation in the GPCR encoded by *frpr-3* completely suppressed the residual egg-laying defect of *egl-47(lf)*;NLP-7 OE animals. *frpr-3(lf); egl-47(lf)*;NLP-7 OE animals retained around 15 eggs *in utero,* similar to wild type (Figure III-3). FRPR-3 belongs to the rhodopsin family of GPCRs and resembles RFamide family of peptide receptors.

Interestingly, FRPR-3 has been previously demonstrated to be activated by peptides encoded by *flp-11* using cellular fluorescent assay in a heterologous system (Mertens et al., 2004). We had already shown that *flp-11* acts in a parallel genetic pathway to *nlp-7* since *nlp-7(lf);flp-11(lf)* double mutants exhibit a strong egg-laying constitutive phenotype (Figure III-3) whereas neither *nlp-7(lf)* or *flp-11(lf)* single mutants show any obvious egg-laying phenotype. We therefore hypothesized that FLP-11 may inhibit egg-laying by acting on the FRPR-3 receptor and this pathway acts in parallel to NLP-7 signaling pathway. In support of this idea, we found that *flp-11(lf)* mutants also completely suppress the residual egg-laying defect of *egl-47(lf)*;NLP-7 OE animals (Figure III-3). Moreover, *nlp-7(lf);frpr-3(lf)* double mutants display a significant egg-laying constitutive phenotype and closely phenocopy *nlp-7(lf);flp-11(lf)* double mutants with regard to the developmental stages of embryos laid during egg-laying (Figure III-3). For instance, *nlp-7(lf);flp-11(lf)* double mutants lay around 58% of their eggs in the early stages of development (1-8 cell stage). Similarly, *nlp-7(lf);frpr-3(lf)* double mutants lay around 45% of early-stage embryos. None of *nlp-7(lf), flp-11(lf)* or *frpr-3(lf)* single mutants show any obvious change in the developmental stage of embryos laid. Together, these results indicate that FLP-11 peptides may inhibit egg-laying by acting through the FRPR-3 receptor and likely act in a parallel genetic pathway with NLP-7 signaling.

To understand where FRPR-3 is endogenously expressed, we used a reporter construct in which coding sequence for the fluorescent protein mCherry preceded by the SL2 splice leader was added to the native *frpr-3* genomic locus that included ~10kb of promoter and the genomic sequence. This bicistronic vector drives expression of the precursor and the mCherry reporter under the control of native regulatory sequences. By

examining mCherry fluorescence, we found that *frpr-3* was expressed in several neurons near the head and a few neurons in the tail region. However, no detectable mCherry fluorescence was observed near the vulva (not shown).

A forward genetic screen to identify downstream regulators of NLP-7 signaling

As described previously, NLP-7 OE animals produced a robust egg-laying defective phenotype. We used this as a tool to identify downstream signaling components that might be mediating the inhibitory effects of NLP-7. We had previously demonstrated that *egl-47(lf)* partially suppressed the egg-laying defect of NLP-7 OE. Therefore, to identify additional suppressors of NLP-7 overexpression induced egg-laying defect, we performed a forward genetic screen using the *egl-47(lf);*NLP-7 OE as the starting strain. *egl-47(lf);*NLP-7 OE animals exhibited an egg-laying defective phenotype that was intermediate between wild type and NLP-7 OE animals. We mutagenized *egl-47(lf);*NLP-7 OE animals and screened their F2 progeny for suppression of the egg-laying defect. Of the animals screened, we found one suppressor (*uf166*) that showed the strongest and most consistent suppression of the egg-laying defect of *egl-47(lf);*NLP-7 OE animals. We quantified the number of eggs *in utero* as a measure of egg-laying. *uf166;egl-47(lf);*NLP-7 OE animals retained around 13 eggs in the uterus, similar to wild type and significantly less than *egl-47(lf);*NLP-7 OE animals (Figure III-4). We genetically isolated the suppressor (*ufIs166*) from *egl-47(lf)* mutation and NLP-7 OE array (*ufIs118*) and found that it displayed a strong egg-laying constitutive phenotype (not shown). *uf166* animals retained around 5 eggs *in utero* compared to 15 eggs in the wild type. Reduction in the number of eggs *in utero* may also indicate reduced production of eggs. To address this

question, we analyzed the developmental stage of embryos laid by *uf166* animals and found that there was a significant increase in the fraction of early stage embryos. This suggests that animals carrying the *uf166* mutation displayed an increase in the rate of egg-laying. It would be crucial to map the position of the allele in the genome and identify the mutation by whole genome sequencing. Identifying the genetic suppressor allele would likely lead to uncovering a key signaling component involved in NLP-7 mediated inhibition of egg-laying behavior.

A loss-of-function mutation in the potassium chloride cotransporter KCC-2 completely suppresses the egg-laying defect of NLP-7 OE

Prior work had demonstrated that a loss-of-function mutation in the gene encoding a potassium chloride cotransporter *kcc-2,* suppressed the egg-laying defect caused by a gain-of-function mutation in *egl-47,* thus indicating the possibility that EGL-47 might act as a chloride channel or may activate some other chloride channel(s) (Tanis et al., 2009). Interestingly, we had demonstrated that a loss-of-function mutation in *egl-47* partially suppressed the egg-laying defect of NLP-7 OE (Figure III-2A). To test the possibility that the actions of NLP-7 might be dependent on chloride gradient, we tested egg-laying behavior in *kcc-2(lf);*NLP-7 OE animals. Strikingly, we found that loss-of-function mutation in *kcc-2* completely suppressed the egg-laying defect of NLP-7 OE animals (Figure III-5). *kcc-2(lf);*NLP-7 OE animals retain similar number of eggs *in utero* as *kcc-2(lf)* mutants (Figure III-5A). A previous study had shown that *kcc-2(lf)* mutants were egg-laying constitutive and also were defective for egg production (Tanis et al., 2009). To test whether the suppression of egg-laying defect of NLP-7 OE animals by *kcc-2(lf)* mutants

was not due to decreased egg production, we assessed the developmental stage of embryos laid by *kcc-2(lf);*NLP-7 OE animals. *kcc-2(lf)* mutants lay ~40% of their eggs in early stages (1-8 cell stage) of development (Figure III-5B). We observed that *kcc-2(lf);*NLP-7 OE animals laid a similar fraction of early stage embryos (Figure III-5C) indicating that *kcc-2* mediated suppression of NLP-7 OE egg-laying defect was not due to decreased egg production. Together, our results suggest that the inhibitory actions of NLP-7 might, at least in part, dependent on the chloride gradient of the target cell.

Discussion

Neuropeptides play a central role in establishing stable behavioral states. However, elucidating the signaling machinery that mediate the actions of neuropeptides has remained a challenge. Identifying the signaling components involved in neuropeptide regulation of behavior would allow us to gain a fundamental understanding of how specific neuropeptide signaling pathways alter the activity of neural circuits at a cellular and molecular level. While previous works have demonstrated several potential mechanisms of neuropeptide action on neural circuit functioning, dissecting neuropeptide signaling mechanisms *in vivo* has largely remained a challenge. Here we have made an attempt to unravel the downstream signaling components involved in neuropeptide modulation of egg-laying behavior. By using powerful genetics, we have been able to identify some of the signaling components that mediate the *in vivo* actions of specific neuropeptides in the context of circuits where they are endogenously released.

Our study has focused on identifying the candidate receptor(s) involved in mediating the inhibitory effects of the neuropeptides NLP-7 and FLP-11 on egg-laying behavior. We had previously demonstrated that the coordinated actions of NLP-7 and FLP-11 establish the temporal organization of egg-laying by setting the duration of inactive phases (see Chapter II). These effects are, at least in part, achieved by regulating serotonergic transmission between the HSN motorneurons and vulval muscles. However, our findings have been limited by the lack of potential receptor(s) that are involved in NLP-7 and FLP-11 signaling. Here, we have performed a candidate genetic suppressor screen using candidate GPCRs that have been predicted to act as neuropeptide receptors. So far, we have identified the GPCRs encoded by the *npr-7* and *npr-8* genes that partially

suppress the egg-laying defect of NLP-7 overexpressing animals. The corresponding ligands for NPR-7 and NPR-8 have not been identified yet. It is possible that these receptors may mediate the inhibitory effects of NLP-7 on the egg-laying circuit. Thus, it would be interesting to see whether NLP-7 peptides could activate these GPCRs in a heterologous system. In addition, determining the cells which express these receptors by examining their respective expression patterns would allow us to elucidate the site(s) of action for NLP-7 peptides. We have also found that FRPR-3, a GPCR that is activated by FLP-11 peptides in human embryonic kidney (HEK) cells, also suppressed the egg-laying defect of NLP-7 OE animals. This raises the interesting possibility that FRPR-3 may also act as one of the potential receptors for NLP-7 mediated inhibition of egg-laying. It remains to be seen whether NLP-7 peptides could activate FRPR-3 in a heterologous system. Nevertheless, the genetic suppression of NLP-7 OE egg-laying defect by both *flp-11* and *frpr-3* likely indicates the existence of a genetic pathway mediated by FLP-11 peptides acting via the FRPR-3 receptor that acts in parallel to the NLP-7 signaling pathway. Unlike FRPR-3 mediated suppression of egg-laying defect of NLP-7 OE animals, FRPR-4, which had also been shown to act as a potential FLP-11 receptor, do not show any obvious effect (Figure III-3), suggesting that the effects are specific to FRPR-3. There are two other candidate receptors of FLP-11 peptides – NPR-4 and NPR-22. However, it remains to be seen whether these receptors are also involved in FLP-11 mediated control of egg-laying.

Our genome-wide EMS mutagenesis screen has identified a candidate that also suppressed the residual egg-laying defect of *egl-47(lf);*NLP-7 OE animals. Genetic isolation of the suppressor has revealed that it exhibits an egg-laying constitutive

phenotype. We had previously shown that loss-of-function in *goa-1* also suppressed the residual egg-laying defective phenotype of *egl-47(lf);*NLP-7 OE animals. To test whether the identified suppressor contains a mutation in the *goa-1* gene, we sequenced the genomic locus of *goa-1* from the start codon to stop codon. Sequencing analysis did not reveal any mutation within that region. Although we cannot rule out the possibility of the suppressor harboring any mutation in *goa-1* promoter or regulatory sequences downstream of the stop codon, our results indicate that the effects of the suppressor allele in reversing the egg-laying defects of *egl-47(lf);*NLP-7 OE animals are not caused by a loss-of-function mutation in the *goa-1* coding sequences. It would be interesting to perform whole-genome sequencing to reveal the identity of the mutant allele. This would potentially allow us to uncover one of the molecular candidates involved in neuromodulation of egg-laying mediated by NLP-7 and/or FLP-11.

Materials and Methods

Strains

All nematode strains were maintained at 20°C on agar nematode growth media plates seeded with *E. Coli* OP50. The wild type reference animals for all cases are the N2 Bristol strain. The following strains were used or generated in this work: IZ1236: *ufIs118 [Pnlp-7::nlp-7::nlp-7 3'UTR, Plgc-11::GFP],* RB850: *egl-47(ok677)V*, IZ1235: *egl-47(ok677);ufIs118,* IC683: *npr-9(tm1652),* IZ2188: *npr-9(tm1652);egl-47(ok677);ufIs118,* XA3702: *npr-2(ok419),* IZ2202: *npr-2(ok419);egl-47(ok677);ufIs118,* RB1393: *npr-5(ok1583),* IZ2203: *npr-5(ok1583);egl-47(ok677);ufIs118,* IZ1882: *frpr-3(ok3302),* IZ2204: *frpr-3(ok3302);egl-47(ok677);ufIs118,* RB1325: *npr-10(ok1442),* IZ2232: *npr-10(ok1442);egl-47(ok677);ufIs118,* RB1329: *npr-8(ok1446),* IZ2217: *npr-8(ok1446);egl-47(ok677);ufIs118,* RB1837: *frpr-4(ok2376),* IZ2260: *frpr-4(ok2376);egl-47(ok677);ufIs118,* RB761: *npr-7(ok527),* IZ2259: *npr-7(ok527);egl-47(ok677);ufIs118,* IZ1005: *ckr-1(ok2502),* IZ1446: *ckr-1(ok2502);egl-47(ok677);ufIs118,* RB799: *npr-11(ok594),* IZ2324: *npr-11(ok594);egl-47(ok677);ufIs118,* MT16665: *egl-6(n4536),* IZ2413: *egl-6(n4536);egl-47(ok677);ufIs118,* IZ1273: *kcc-2(vs132);ufIs118*, LX999: *kcc-2(vs132).*

Molecular Biology and Transgenes

NLP-7 overexpression: The NLP-7 OE strain (*ufIs118*) was generated by microinjection and subsequent X-ray integration of a 5104 bp PCR product (injected at 100 ng/μl) containing the *nlp-7* promoter (about 3.5 kb), genomic locus and the 3'UTR (-3448 bp to

+1656 bp relative to the transcriptional start) along with pHP6 (*Plgc-11::GFP,* 50 ng/μl) as coinjection marker. The integrated strain was outcrossed five times with wild type.

Behavioral assays

Quantification of eggs in uterus. Age-matched adults were obtained by collecting late fourth larval stage (L4) animals and culturing at 20°C for 30 hrs. For each strain analyzed, animals were individually dissolved in 25% sodium hypochlorite, and their eggs, which survived because of their protective eggshells, were quantified.

Embryo staging assay. To score the developmental stage of newly laid eggs, age-matched adults (30 hrs after the late L4 stage) were transferred to fresh nematode growth medium plates (15 animals per plate), allowed to lay eggs for 30 mins and removed. Eggs laid on the plates were examined by a high power dissecting microscope and categorized as described in Ringstad and Horvitz, 2008.

EMS mutagenesis screen

Egg-laying defective *egl-47(lf);*NLP-7 OE animals were mutagenized with 50mM ethyl methanesulphonate (Brenner, 1974). F2 progeny of ~1500 mutagenized animals were collected as fourth larval stage (L4) animals, washed twice with M9 buffer and transferred to fresh NGM agar plates. After ~36 hrs following the L4 stage, the animals were screened for suppression of the egg-laying defect and were individually transferred to single plates. The progeny from each plate were individually treated with 25% sodium hypochlorite and the eggs retained in the uterus were quantified.

Microscopy

DIC imaging

Worms were mounted on agarose pads and immobilized with 0.3 M sodium azide. All images were obtained from staged young adult animals (~30hrs after the L4 stage). Images were acquired using a Zeiss Axioskop 2 microscope system and LSM Pascal 5 imaging software (Zeiss).

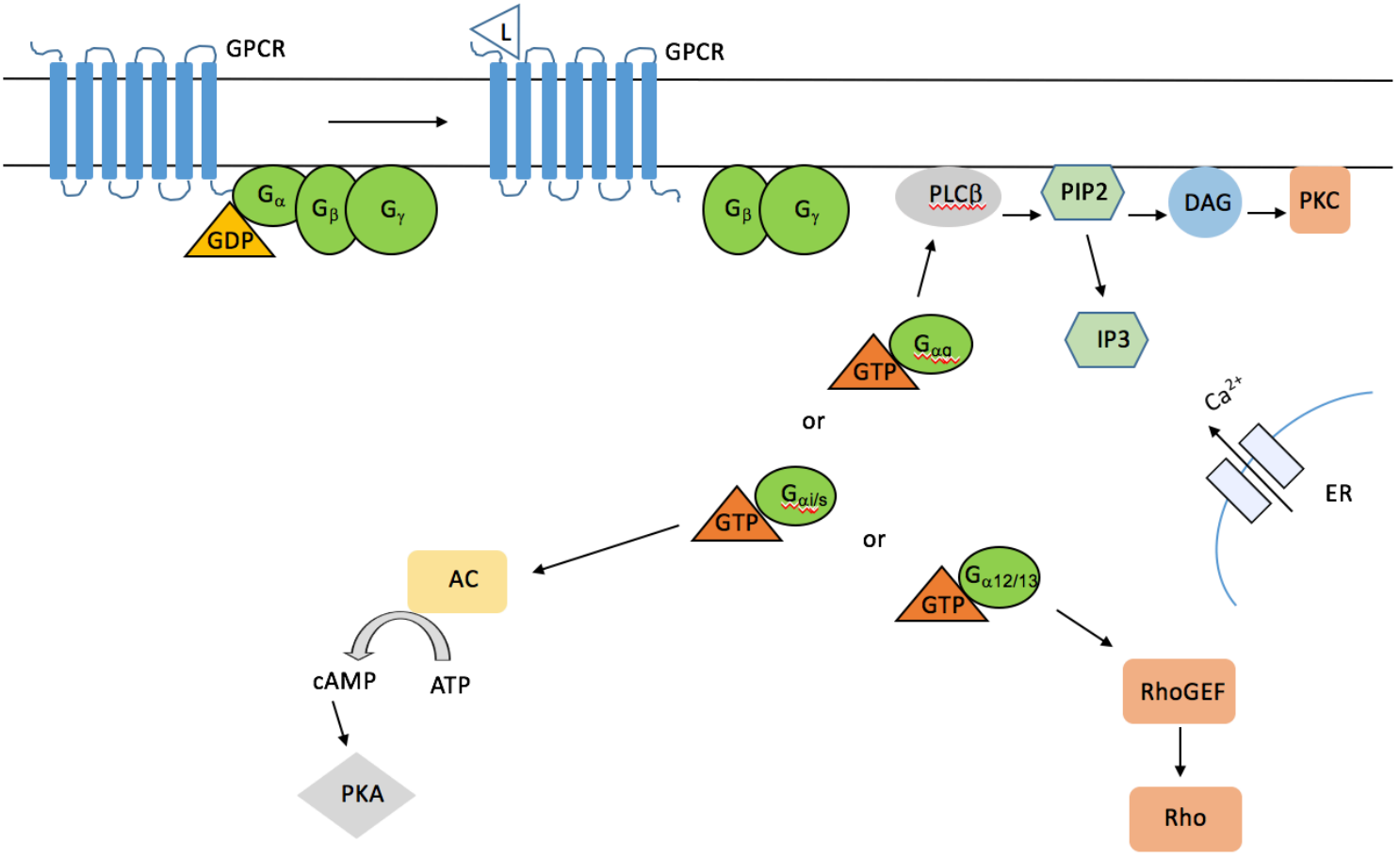
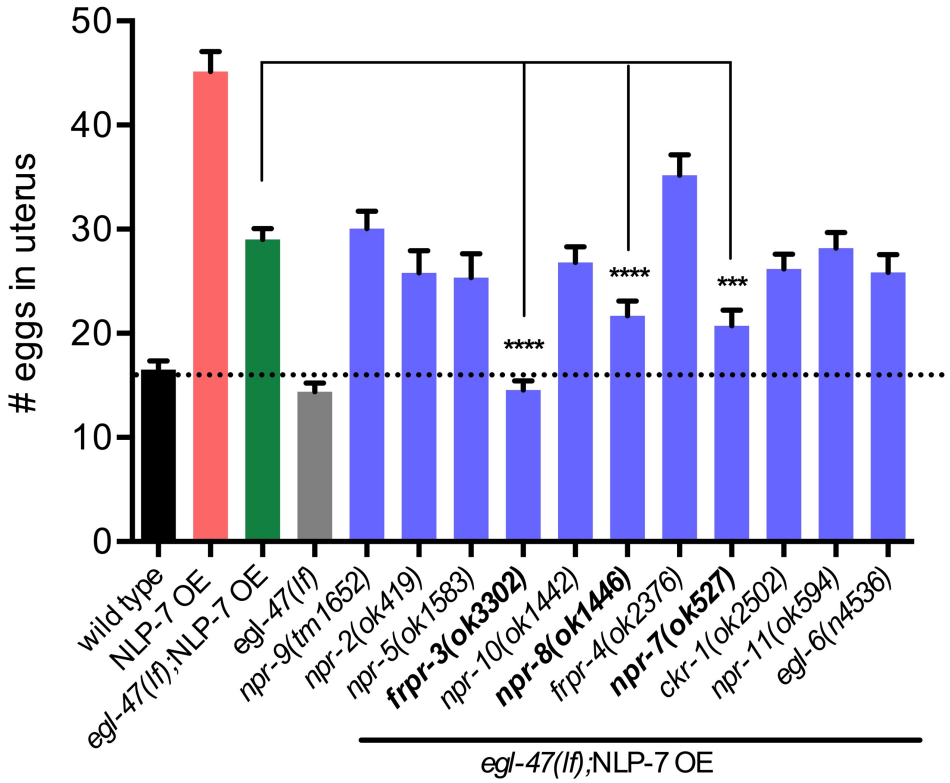


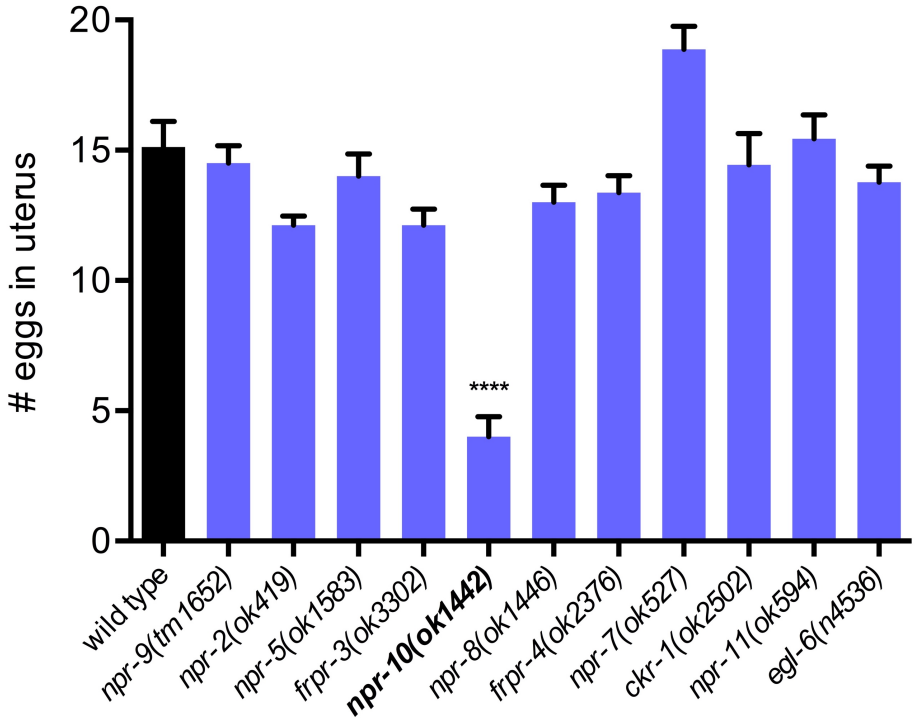
Figure III-1. The classical GPCR signaling pathway. G-protein signaling is highly conserved between *C. elegans* and mammals. On ligand binding, the receptor changes conformation followed by the exchange of GDP to GTP by guanine nucleotide exchange factor (GEF). The heterotrimeric G-protein dissociates from the receptor and splits into a

Ga‐GTP and a Gbg subunit. The Ga‐GTP subunit binds to different downstream effectors depending on the Ga subtype as indicated. G protein is terminated by internalization of the GPCR that is initiated by phosphorylation of the receptor by GPCR kinases.

A


eggs in uterus
50
40
30
20
10
0
****
****
***
wild type
NLP-7 OE
egl-47(lf);NLP-7 OE
egl-47(lf)
npr-9(tm1652)
npr-2(ok419)
npr-5(ok1583)
frpr-3(ok3302)
npr-10(ok1442)
npr-8(ok1446)
frpr-4(ok2376)
npr-7(ok527)
ckr-1(ok2502)
npr-11(ok594)
egl-6(n4536)
egl-47(lf);NLP-7 OE

B


eggs in uterus
20
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wild type
npr-9(tm1652)
npr-2(ok419)
npr-5(ok1583)
frpr-3(ok3302)
npr-10(ok1442)
npr-8(ok1446)
frpr-4(ok2376)
npr-7(ok527)
ckr-1(ok2502)
npr-11(ok594)
egl-6(n4536)

Figure III-2. A candidate suppressor screen to identify receptor(s) for the neuropeptides NLP-7 and FLP-11.

(A) Schematic for the genetic strategy of the suppressor screen. See text for details. (B) Quantification of eggs *in utero* for wild type, NLP-7 OE*, egl-47(lf);*NLP-7 OE and *gpcr(lf)* mutants in the *egl-47(lf);*NLP-7 OE genetic background. Bars represent mean ± SEM for each genotype. n≥15 for all genotypes. **** p<0.0001, *** p<0.001 ANOVA with Sidak's post-hoc test. (C) Quantification of eggs *in utero* for wild type and GPCR loss-of-function mutants used as candidates for the screen. Bars represent mean ± SEM for each genotype. n≥8 for all genotypes. **** p<0.0001, *** p<0.001 ANOVA with Sidak's post-hoc test.

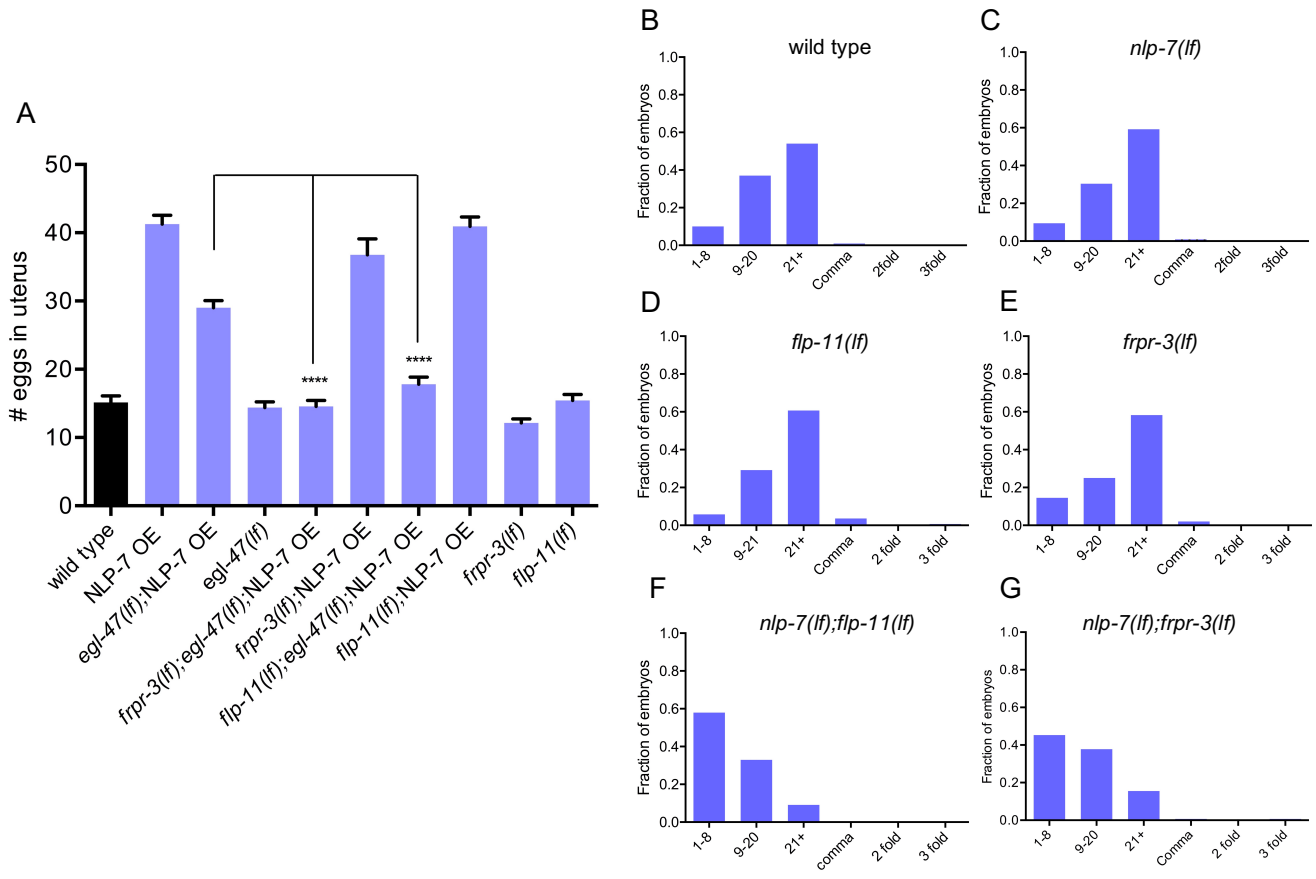

Figure III-3. FLP-11 may act through its receptor FRPR-3 in a parallel genetic pathway to NLP-7 signaling.

(A) Quantification of eggs *in utero.* Loss-of-function mutations in *flp-11 and frpr-3* suppress the residual egg-laying defect of *egl-47(lf)*;NLP-7 OE animals. Bars represent mean ± SEM for each genotype. n≥15 for all genotypes. **** p<0.0001, ANOVA with Sidak's post-hoc test. (B) Quantification of the developmental stage of embryos laid by animals of the indicated genotype. *nlp-7(lf);frpr-3(lf)* double mutants laid significantly higher percentage of embryos in the 1-8 cell stage than *nlp-7(lf)* or *frpr-3(lf)* single mutants. p<0.0001, Fisher's exact test.

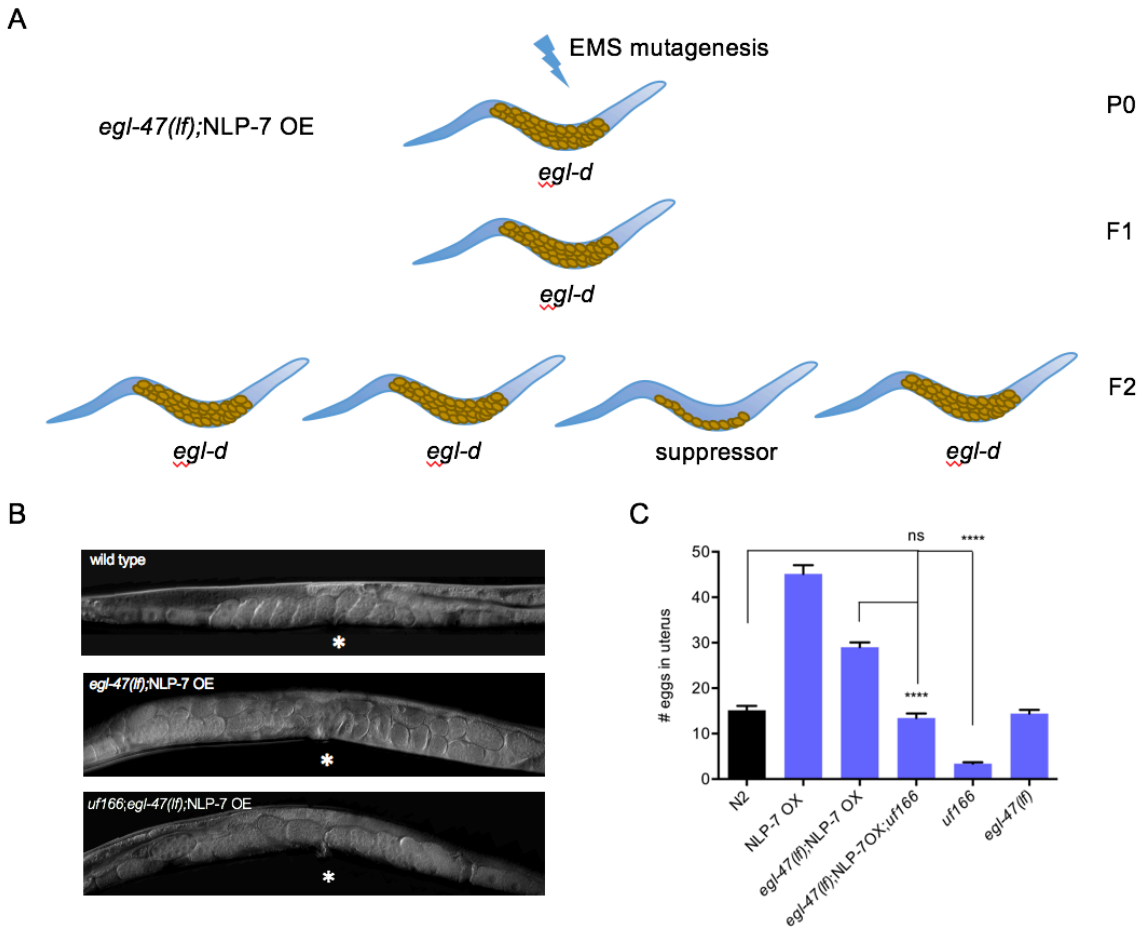

Figure III-4: An EMS screen to identify downstream effectors of NLP-7 signaling.

(A) Schematic showing the strategy for the EMS screen (see text for details). (B) DIC images of wild type, *egl-47(lf)*;NLP-7 OE and *uf166;egl-47(lf)*;NLP-7 OE animals. Note the decreased number of eggs in the uterus of *uf166;egl-47(lf)*;NLP-7 OE animals. Asterisks denote position of the vulva. (C) Quantification of eggs *in utero* for the genotypes indicated. *uf166;egl-47(lf)*;NLP-7 OE animals retain significantly fewer eggs in uterus compared to *egl-47(lf)*;NLP-7 OE. *uf166* animals are egg-laying constitutive. Bars represent mean ± SEM for each genotype. n≥15 for all genotypes. **** p<0.0001, ANOVA with Sidak's post-hoc test.

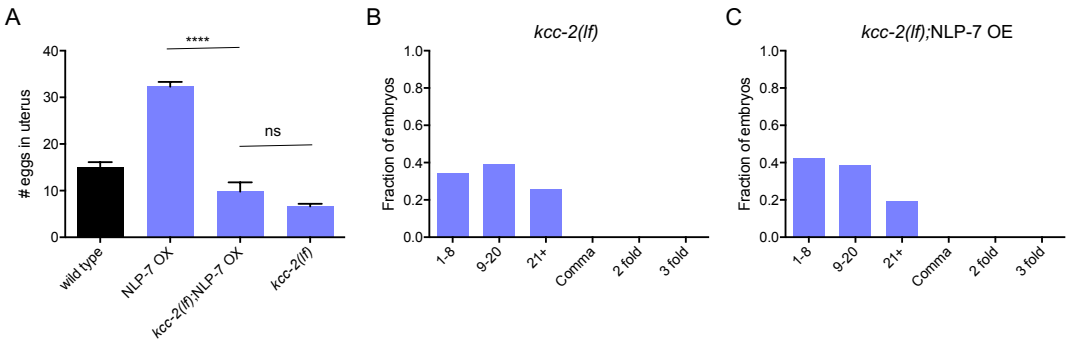

Figure III-5: A loss-of-function in *kcc-2* suppresses the egg-laying defect of NLP-7 overexpression. (A) Quantification of eggs *in utero* for the genotypes indicated. Kcc-2(lf);NLP-7 OE animals retain significantly fewer number of eggs *in utero* compared to NLP-7 OE animals. Bars represent mean ± SEM for each genotype. n≥15 for all genotypes. (**** p<0.0001, ANOVA with Sidak's post-hoc test. ns = not significant). (B,C) Distribution of the developmental stages of eggs laid by *kcc-2(lf)* and *kcc-2(lf)*;NLP-7 OE animals. *kcc-2(lf)*;NLP-7 OE animals laid similar proportion of eggs in the early stages of development (1-8 cell stage) as *kcc-2(lf)* single mutants.

CHAPTER IV: DISCUSSION

Neuromodulatory control of behavioral states

To maximize their chances of survival, animals must constantly switch between different behavioral states and rapidly modify their durations in response to the changing environment as well as internal states. One of the most well-studied examples is sleep-wake behavior. Sleep can be further subdivided into two phases – rapid eye movement sleep (REM) characterized by vivid dreams and non-REM sleep (NREM) with decreased sensation (Hobson, 2005). Similarly, wakefulness is also composed of quiet, inattentive and aroused, vigilant states. The underlying neural mechanisms controlling transitions between these component states are molecularly represented by neuromodulators. Neuromodulators enable neural circuits to rapidly change their activity patterns in a context-dependent manner. Typically, through modifications of neuronal excitability, dynamics and synaptic efficacy between components of a circuit, neuromodulators enable a fixed complement of neurons within a circuit to generate alternate activity patterns and consequently, give rise to opposing behavioral outcomes. It is crucial to gain a fundamental understanding of neuromodulatory mechanisms operating *in vivo* to establish distinct behavioral states and coordinate transitions between them.

The relative simplicity of its nervous system and amenability to powerful genetic analyses makes *C. elegans* a useful model to study neuromodulation. The behavioral program associated with egg-laying in *C. elegans* is composed of alternating behavioral states – active phase and inactive phase. However, the mechanisms that govern the timing of egg-laying had remained unaddressed. Previous work had shown that egg-laying behavior is susceptible to changes in environmental conditions (Fenk and de Bono,

2015; Ringstad and Horvitz, 2008; Waggoner et al., 2000; Zhang et al., 2008). We demonstrate that egg-laying behavior is subject to neuromodulatory control even in the absence of any external stimuli. In Chapter II, we show that a group of neurosecretory cells (uv1) establish the timing of egg-laying events under favorable conditions by modulating the activity of a core neural circuit. Our study has also demonstrated a requirement of local neuromodulation in regulating egg-laying. We have identified the molecular candidates that mediate the neuromodulatory effects of uv1 cells. Two neuropeptides encoded by the *nlp-7* and *flp-11* genes are released by the uv1 cells and act locally to inhibit serotonergic transmission between the HSN motorneurons and vulval muscles, thereby preventing premature transitions from the inactive phase to the active phase of egg-laying.

Our observations indicate that the inhibitory actions of uv1 cells on egg-laying behavior are mediated by NLP-7 and FLP-11 peptides. However, the roles of these peptides on uv1 mediated inhibition are not complete (Figure II-2, II-3D), suggesting that other signaling molecules may also play a role. As described previously (Alkema et al., 2005), the uv1 cells also express the monoamine transmitter tyramine and exogenous tyramine could halt egg-laying activity. It is possible that tyramine and these neuropeptides act cooperatively in the inhibition of egg-laying. We did some preliminary experiments to test this idea. We have quantified eggs *in utero* for *tdc-1; nlp-7; flp-11* triple mutant compared with the *nlp-7; flp-11* double or either single mutant (Figure IV-1). We do not observe significant enhancement with mutations in *tdc-1* or *lgc-55* (candidate receptor for tyramine) using this approach. However, we are somewhat hesitant to draw firm conclusions from these negative data. As tyramine likely acts through the ligand-

gated channel *lgc-55* to affect egg-laying (Collins et al., 2016), it seems logical that the actions of tyramine may occur on a more rapid time scale that would not necessarily be reflected in our analysis. We therefore believe that a more complete exploration of tyramine interactions with neuropeptide signaling is warranted. A detailed analysis of the temporal dynamics of egg-laying may reveal an interesting synergy between the two modes of signaling systems.

Our results also indicated at least one of the potential mechanisms by which inhibition of serotonin release is achieved. We show that altering levels of the neuropeptide NLP-7 leads to changes in synaptic vesicle abundance in the HSN motorneurons. Although several plausible mechanisms could be responsible for such changes (see Chapter II Discussion), it would be interesting to see whether these effects are directly mediated by neuropeptides or whether they are a result of intrinsic feedback mechanism(s) in the HSN motorneurons in response to chronic changes in its activity. One way of testing this idea would be to temporally regulate the actions of these peptides by expressing them in an acute fashion under the control of a heat shock promoter and monitoring changes in synaptic vesicle abundance with time measured from the onset of heat shock. Another potential mechanism by which these peptides might inhibit HSN activity is by reducing calcium flux into the HSNs. According to prior reports, calcium activity in the HSNs are tightly correlated with egg release from the vulva (Zhang et al., 2008, Collins et al., 2016) and there is a significant reduction in the frequency of HSN calcium spikes during inactive periods of egg-laying compared with active phases (Collins et al., 2016). It is possible that neuropeptides encoded by *nlp-7* and *flp11* negatively regulate calcium activity in the HSNs during inactive phases of egg-laying and help to

maintain the appropriate duration of inactive phases. Measuring HSN calcium activity *in vivo* in freely behaving animals would be a good way to test this idea. Our genetic analyses in Chapter II indicate that the effects of the peptides may not be limited to the HSNs. Therefore, it would also be intriguing to see whether peptidergic signaling mediated by NLP-7 and FLP-11 also affects the activities of other cells in the circuit, such as vulval muscles or the VC motorneurons. Addressing these questions would potentially provide useful insights into mechanisms underlying changes in levels of neurotransmission mediated by neuropeptidergic signaling under *in vivo* conditions.

Our findings in Chapter II were limited by the lack of knowledge about the potential neuropeptide receptors and downstream signaling components that mediate the actions of NLP-7 and FLP-11. In Chapter III, we have identified at least some of the molecules that may act as candidate receptors for the two neuropeptides. A crucial step forward would be to identify the cells where the receptors act to mediate the inhibitory actions of NLP-7 and FLP-11 on the egg-laying circuit. Deciphering the sites of action of the neuropeptides would allow us to gain a deeper understanding of the cellular mechanisms underlying *in vivo* actions of neuropeptides in modulating neural circuit activity.

Based on our findings in Chapter II and III, we propose a model where the coordinated actions of neuropeptides encoded by *nlp-7* and *flp-11* negatively regulate the activity of the motor circuit responsible for egg-laying behavior. Our powerful genetic analyses have provided some useful insights into the possible mechanism(s) of action of these peptides. We hypothesize that NLP-7 peptides inhibit the HSNs by at least two parallel mechanisms (Figure IV-2). One pathway involves activation of the seven trans-membrane protein EGL-47, which through its predicted role as a regulator of chloride

channel(s), increases chloride flux into the HSNs resulting in its hyperpolarization and subsequent failure to promote egg-laying active phases. The other pathway involves activation of a yet unknown GPCR that is coupled to the $G_{\alpha o}$ subunit GOA-1 which has been reported to inhibit serotonin release from the HSNs, thereby preventing the animal to enter active phases prematurely.

Complexity of neuromodulatory networks in vivo

Neuromodulatory strategies are remarkably complex – single modulators act on multiple neurons and activate multiple receptors or single neurons can be modulated by multiple neuromodulators. Work done on neural circuits of crustaceans and mollusks for more than 40 years have shed light on the complexity of neuromodulatory signaling. In almost every situation, the activity of a single neural circuit is subject to the modulatory effects of several modulators making it extremely difficult to identify all the modulatory inputs to a given circuit. Therefore, decoding the rules of modulation *in vivo* in the context of complex neural circuits in vertebrates has remained a major challenge. In addition, linking the actions of neuromodulators *in vivo* with generation of distinct behaviors has largely remained unanswered.

One of the reasons why *C. elegans* has emerged as a useful model to study neuromodulation is the simplicity of its nervous system and the known connectivity diagram. Having a full knowledge of the anatomical wiring of the nervous system serves as a platform to study specific neuromodulatory mechanisms responsible for alterations of activity within a defined circuit. Studies on neuromodulation in *C. elegans* have

demonstrated some significant violations of the one neuron – one behavior rule. Neuromodulators have been shown to alter circuit composition in a context dependent manner and enable distinct populations of neurons to participate in a single behavior under different conditions. For example, octanol avoidance behavior is mediated solely by the ASH neurons under well-fed conditions. In contrast, starvation leads to distribution of this avoidance behavior between ASH, AWB and ADL neurons (Chao et al., 2004). Several neuromodulators such as serotonin, dopamine, tyramine, octopamine and several neuropeptides have been implicated in this change in circuit composition (Horvitz et al., 1982; Wragg et al., 2007; Mills et al., 2011). Single neuromodulatory signaling pathways have also been demonstrated to lead to multiple behavioral outputs. For example, neuropeptide signaling mediated by the NPR-1 receptor that regulates aerotaxis also affects a second behavior, aggregation of animals into feeding groups or social feeding (Chang et al., 2006; Macosko et al., 2009; Rogers et al., 2006; de Bono and Bargmann, 1998). The functional diversity of neuromodulatory pathways arises, in part, due to utilization of distinct receptors by the same neuromodulator in different subsets of neurons. For instance, part of the effect of serotonin on avoidance responses is mediated by a G-protein coupled serotonin receptor expressed in ASH sensory neurons whereas another part is mediated by a serotonin gated ion channel in interneurons (Harris et al., 2009). Further, the neuropeptide FLP-18 acts on the NPR-4 receptor in AVA and RIV neurons to modulate chemosensory responses and foraging behavior, whereas it regulates dauer formation by activation of NPR-5 in ASJ neurons (Cohen et al., 2009). The actions of multiple neuromodulators may also converge onto a single receptor to generate a particular behavior. For example, the neuropeptides FLP-10 and FLP-17 are

released from the head sensory neurons and act on the EGL-6 receptor in a motorneuron to inhibit egg-laying behavior (Ringstad and Horvitz, 2008). These studies indicate that neuromodulators select a subset of neurons from a large network of anatomically-specified connections. Structurally hardwired circuits have the potential for generating different behaviors, but the neuromodulatory state defines which potential is available at a given time under a specific set of conditions. Our findings in Chapter II reveal that the anatomically defined neural circuit for *C. elegans* egg-laying is sufficient to promote successful deposition of eggs, but neuromodulatory signaling (mediated by the NLP-7 and FLP-11 neuropeptides) refines activity within the circuit and enables the same circuit to adopt distinct functional configurations to generate two opposing behavioral outcomes – the active phase and inactive phase. Our results also point towards a specialized role for neurosecretory cells as sites for neuromodulator release and demonstrate a local requirement of peptide release to alter circuit function and lead to profound changes in the timing of a behavioral program.

Future studies on neuromodulation

Anatomical reconstructions and ultrastructural analyses aimed at understanding the full set of neuronal connections and gaining knowledge about the rules for synaptic connectivity have proven and will prove invaluable for understanding brain function. There is no other way of dissecting the functions of circuits without knowing their connectivity diagrams. However, it is not sufficient to interpret differential activity patterns within the brain. It would not explain the modifications in circuit composition and dynamics that arise

from neuromodulatory actions. Most neuromodulators are non-synaptically released and may diffuse over long distances to act on distant targets, thus largely getting ignored in the analysis of the connectome. This, coupled to the immense complexity of neuromodulatory networks have limited our understanding about neuromodulation at the most basic level.

Gaining a complete knowledge of brain function will require the connectivity diagram that is supplemented with detailed information of all neurotransmitters and neuromodulators expressed in each neuron. Identification of their receptors and downstream effectors along with a fundamental understanding about their functions will also be required. It will be crucial to be able to simultaneously record the electrical activities of multiple components within a circuit at a specific modulatory state. Computational models would be necessary to understand or predict multiple behavioral outputs from single circuits. Finally, it will require the use of suitable model systems where *in vivo* actions of specific neuromodulatory signaling pathways could be correlated with behavioral flexibility in the context of the environment and internal modulatory states. Elucidating and comparing the general principles of modulation across species would potentially lead us to understand the fundamental rules of neuromodulation that govern the adaptability of circuits in response to changing contexts.

In this dissertation, I demonstrate the *in vivo* actions of specific neuromodulatory systems in shaping alternate behavioral outcomes by studying neuropeptide signaling in the context of the relatively simple circuit responsible for *C. elegans* egg-laying behavior. The work presented in Chapter II, demonstrates how neuropeptidergic signaling is encoded within a neural circuit under *in vivo* conditions. It indicates a role for

neurosecretory cells (uv1) in locally modulating the activity of motorneurons (HSNs) and regulating serotonergic transmission within the circuit. In Chapter II, I also identify the molecular candidates (NLP-7 and FLP-11) that mediate the modulatory actions of uv1 cells and shape the temporal organization of egg-laying by setting the duration of quiescent periods (inactive phases) in the behavioral program. In Chapter III, I elucidate the potential receptors and downstream signaling components that may be mediating the action of the neuropeptides NLP-7 and FLP-11 in modulating circuit activity. This dissertation uncovers principles of neuromodulatory actions operating *in vivo* and links them to distinct behavioral outcomes. Understanding the basic rules that govern neuromodulation in simple model organisms will significantly contribute towards deciphering modulatory principles in more complex circuits and attaining a complete knowledge of neural circuit mechanisms underlying distinct behavioral outcomes.

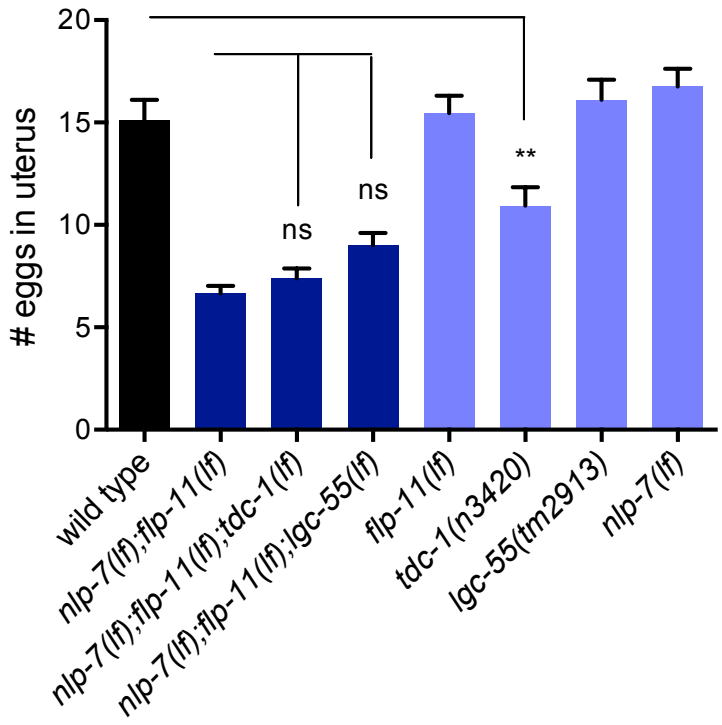

Figure IV-1. Genetic interactions between tyramine and neuropeptides.

Quantification of eggs *in utero* for the genotypes indicated. *tdc-1(lf)* and *lgc-55(lf)* mutants fail to enhance the constitutive egg-laying of *nlp-7(lf);flp-11(lf)* double mutants. Bars represent mean ± SEM for each genotype. ** $p < 0.01$, ns - not significant.

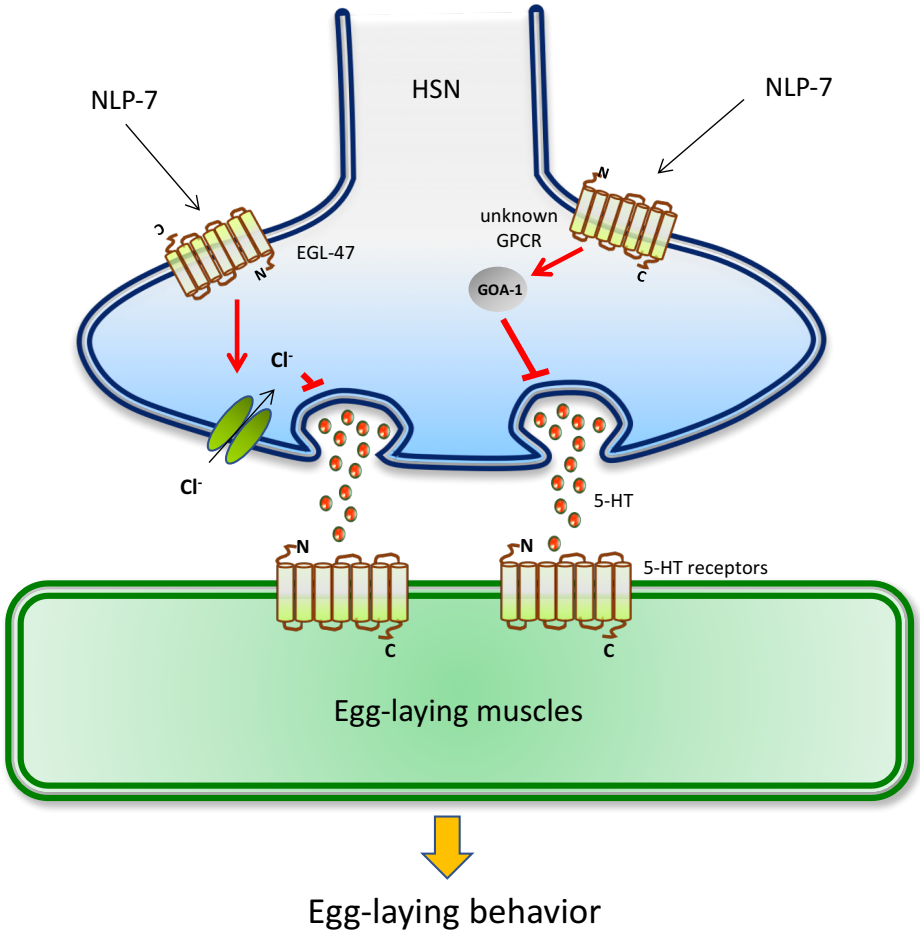

Figure IV-2. Possible pathways of NLP-7 mediated inhibition of HSN activity and egg-laying behavior. NLP-7 peptides may inhibit HSN activity through EGL-47 that in turn, may regulate the activity of chloride channels resulting in chloride influx and subsequent hyperpolarization of the HSNs. In a parallel genetic pathway, NLP-7 may bind to a yet unknown $G_{\alpha o}$ coupled GPCR and operate through GOA-1 to inhibit serotonin release from the HSNs. These two parallel genetic pathways operate redundantly to inhibit HSN activity and promote periods of quiescence in the egg-laying behavioral program.

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